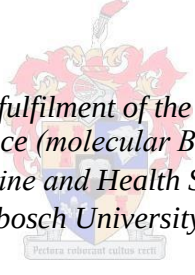
The impact of B-cell activation on the T-cell immune response to *Mycobacterium tuberculosis*

by
Ilana Claudia van Rensburg



Thesis presented in partial fulfilment of the requirements for the degree of Master of Science (molecular Biology) in the Faculty of Medicine and Health Sciences at Stellenbosch University

Supervisor: Dr. André G. Loxton

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Declaration

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Ilana Claudia van Rensburg

November 2016

Abstract

The host immune response to tuberculosis is extremely complex, and in order to fully elucidate this, one has to thoroughly study the host response by considering all immune cells and phases of infection. T-cells have always been at the forefront of tuberculosis research due to its production of interferon-gamma and other pro-inflammatory cytokines. However, our increased understanding of B-cells show that these cells display non-humoral functions, such as production of cytokines and the expression of death-inducing ligands. These additional functions indicate that B-cells have a role to play during infection with *Mycobacterium tuberculosis*. We hypothesize that B-cells utilise these additional functions, like the production of cytokine, to modulate T-cell phenotype and function during exposure to *Mycobacterium tuberculosis*.

Individuals with newly diagnosed untreated tuberculosis were recruited for the study, and were followed up during treatment. Controls recruited included healthy exposed/infected and uninfected controls, as well as other lung disease controls. Peripheral blood was collected from all these individuals. In order to evaluate B-cell gene regulation, transcriptional analysis was performed. Secondly, B-cell phenotypic characterization was done using flow cytometry and secreted cytokines evaluated using the Luminex platform. Furthermore, to assess the interaction between B- and T-cells, autologous B- and T-cells were co-cultured under various stimulation conditions, and the cells and cytokine production (Interferon-gamma, Tumour Necrosis Factor-alpha and Interleukin-2) analysed using flow cytometry.

The results indicate that there is decreased activation of B-cells during active tuberculosis, and this is restored during anti-TB treatment. Transcriptional and phenotypic analysis show that there is a decline in FasL expression by B-cells during tuberculosis (compared to controls), and that the expression is enhanced following successful treatment. A potential function underlying FasL regulation could involve the induction of apoptosis of infected T-cells as a means to rid the body of pathogens. Furthermore, co-culture experiments show that B-cells are able to induce cytokine production by both $CD4^+$ and $CD8^+$ T-cells in the context of *Mycobacterium tuberculosis* exposure.

These results could prove significant as B-cells may serve as a target for new vaccines or modified TB treatment. Current interventions are aimed at enhancing T-cell responses in order to combat infection. However, if B-cells are able to modulate T-cell responses, then these cells could be used to indirectly enhance the T-cell responses needed to eliminate the bacteria.

Opsomming

Die gasheer immuun reaksie tot tuberkulose is uiters kompleks, en om dit ten volle te verstaan, moet 'n deeglike studie van die gasheer reaksie gedoen word deur alle immuunselle en fases van infeksie te oorweeg. T-selle was nog altyd aan die voorpunt van tuberkulose navorsing as gevolg van die produksie van interferon-gamma en ander pro-inflammatoriese sitokiene. Met ‘n toename in ons begrip van B-selle is dit duidelik dat hierdie selle nie-humorale funksies vertoon, soos onder andere die produksie van sitokiene en die uitdrukking van die dood induserende ligande. Hierdie bykomende funksies dui aan dat B-selle 'n rol het om te speel gedurende infeksie met *Mikobakterium tuberkulose*. Ons hipotese is dat B-selle hierdie bykomende funksies, soos die produksie van sitokiene gebruik, om T-sel fenotipe en funksie te moduleer tydens blootstelling aan *Mikobakterium tuberkulose*.

Individue met nuut gediagnoseerde onbehandelde tuberkulose was gewerf vir die studie, en is opgevolg tydens behandeling. Gesonde kontrole wat blootgestel/besmet is en onbesmette kontrole, sowel as kontroles met ander longsiekte was ook gewerf. Perifere bloed is gekollekteer vanaf al hierdie individue. Om B-sel geenregulering te evalueer, is transkripsionele analise uitgevoer. Tweedens is B-sel fenotipiese karakterisering gedoen met behulp van vloeisitometrie en die vlakke van geproduseerde sitokiene geëvalueer met behulp van die Luminex platform. Om die interaksie tussen B- en T-selle te bepaal, is hierdie selle in kultuur saamgekweek onder verskillende stimulasie kondisies, en die selle en sitokiene produksie (Interferon-gamma, Tumor Nekrose Faktor-alfa en Interleukien-2) ontleed met behulp van vloeisitometrie.

Die resultate dui daarop dat die aktivering van B-selle afneem tydens aktiewe tuberkulose, en dat dit herstel tydens anti-TB behandeling. Transkripsionele en fenotipiese analise toon dat daar 'n afname in FasL uitdrukking is deur die B-selle tydens tuberkulose (in vergelyking met die kontrole), en dat die uitdrukking bevorder word na suksesvolle behandeling. 'n Potensiële funksie sluit in dat FasL regulasie die inlywing van apoptose van besmette T-selle betrek as 'n manier waardeer die liggaam ontslaee raak van patogene. Verder, kwekings eksperimente toon dat B-selle in staat is om sitokien produksie deur beide $CD4^+$ en $CD8^+$ T-selle in die konteks van blootstelling aan *Mikobakterium tuberkulose kan verander*.

Hierdie resultate dui aan dat B-selle as 'n teiken vir nuwe entstowwe of TB behandeling kan dien. Huidige ingrypings is gemik op die verbetering van T-sel reaksies ten einde infeksie te bekamp.

Indien B-selle in staat is om T-sel reaksies te moduleer, kan hierdie selle gebruik word om die T-sel reaksie indirek te verbeter om sodoende die bakterieë uit te skakel.

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Table of Contents

Declaration i

Abstract ii

Opsomming iii

Acknowledgments v

List of Figures 3

List of Tables 4

List of Abbreviations 5

Chapter 1. Introduction and Literature review 8

1.1 Immunology of Tuberculosis 8

1.2. T-cell activation 9

1.3. B-cell activation and effector functions 10

1.4. Relationship between B- and T-cells 12

1.5. Hypothesis 13

1.6 Aims and Objectives 14

Chapter 2. Successful TB treatment induces B-cells expressing FASL and IL5RA mRNA 16

2.1. Abstract 16

2.2. Introduction 17

2.3. Materials and Methods 18

2.3.1. Participant recruitment and Sample collection 18

2.3.2. Sample preparation, mRNA isolation and Quality Check 19

2.3.3. cDNA synthesis and RT-qPCR 20

2.3.4. Data Analysis 21

2.4. Results 21

2.4.1. Comparison of gene expression between TB, OLD and Control groups 21

2.4.2. Differential gene expression during Anti-TB treatment 23

2.5. Discussion 27

Chapter 3. B-cells with a FasL expressing regulatory phenotype are induced following successful anti-Tuberculosis treatment 30

3.1. Abstract 30

3.2. Introduction 31

3.3. Materials and Methods 32

3.3.1. Ethics Statement 32

3.3.2. Participant recruitment 32

3.3.3. B-cell phenotype analysis using Flow cytometric analysis 33
3.3.4. B-cell gene expression analysis 34
3.3.5. Host marker detection in BAL and PLASMA using LUMINEX platform 35
3.3.6. Statistical analysis 35
3.4. Results 36
3.4.1. Activated B-cells express a $FasL^{+}IL5Ra^{+}$ phenotype following anti-TB treatment 36
3.4.2. Effects of anti-TB treatment on the expression of Regulatory and exhaustion markers by B-cells 37
3.4.3. Expression of genes linked to regulatory phenotype during treatment 40
3.4.4. Changes in BAL-secreted cytokines following anti-TB treatment but not in the plasma of TB cases 41
3.5. Discussion 43
Chapter 4. B- and T-cell interactions in the context of *M.tb* exposure 47
4.1. Introduction 47
4.2. Materials and Methods 48
4.2.1. Ethics statement 48
4.2.2. Participant recruitment and sample collection 48
4.2.3. B- and T-cell co-culture 49
4.2.4. B- and T-cell Flow Cytometry 50
4.2.5. Statistical Analysis 50
4.3. Results 51
4.3.1. Regulatory and Memory B-cell frequencies 51
4.3.2. FasL-expressing B-cells 52
4.3.3. Effector and Regulatory T-cells 54
4.3.4. Relationship between Bregs and Tregs 55
4.3.5. T-cell cytokine production 58
4.4. Discussion 62
Chapter 5. Discussion 67
References 77
Addenda 89

List of Figures

Figure 1.1. Effects of Bregs on Tregs and Effector T-cells during gastric cancer 12
Figure 1.2. Proposed relationship between cytokine-producing B-cells and T-cells 14
Figure 2.1. Differences in mRNA expression between TB and Control groups 23
Figure 2.2. Genes differentially expressed during anti-TB treatment.....25
Figure 2.3. Differential mRNA expression over the course of anti-TB treatment 26
Figure 3.1. The Gating strategy used to identify B-cells of interest 37
Figure 3.2. Frequency of IgM^- and IgM^+ B-cells expressing FASL(CD178)/IL5Ra(CD125) and CD40/PD1 between TB cases and CTRLs 38
Figure 3.3. Frequencies of Regulatory B-cells expressing CD178 and IL5A during treatment ..39
Figure 3.4. Frequencies of Regulatory B-cells expressing CD40 and PD1 during treatment 40
Figure 3.5. Genes differentially expressed during anti-TB treatment.....41
Figure 3.6. Comparison of analyte levels in BAL and Plasma from TB cases at diagnosis and end of treatment 42
Figure 3.7. Comparison of analyte levels in BAL between Ctrls, TB participants at diagnosis and end of treatment. 43
Figure 4.1. Changes in Frequency of Bregs and Bmemory cells. 52
Figure 4.2. Changes in frequency of FasL-expressing B-cells.....53
Figure 4.3. Changes in the frequency of TE and Treg cells 55
Figure 4.4. Correlation between frequency of Breg and TE cells 56
Figure 4.5. Correlation between Frequency of Breg and Treg cells 57
Figure 4.6. Cytokine production by $CD4^+$ T-cells.....59
Figure 4.7. Cytokine production by $CD8^+$ T-cells.....61
Figure 5.1. Proposed effect of FasL-expressing B-cells during active TB disease 71
Figure 5.2. Proposed relationship between Bregs, Tregs and TEs during LTBI.....73
Figure B1. Gating strategy for identifying Breg and Bmemory cells. 90
Figure B2. Gating strategy used to identify FasL-expressing B-cells 91
Figure B3. Gating strategy used to identify Treg and TE cells 92
Figure B4. Gating strategy used for $CD4^+$ and $CD8^+$ T-cell cytokines 93

List of Tables

Table 1. Cell Phenotypes 7

Table 2.2 Clinical and demographic characteristics of study participants 19

Table 2.3. Gene expression differences between TB and Control Groups 22

Table 2.4. Gene expression in TB group over 6 months of treatment 24

Table 3.5. Inclusion and Exclusion criteria for participant recruitment 33

Table 4.6. Culturing conditions for B- and T-cells 49

List of Abbreviations

(As it appears in text)

ANOVA	Analysis of Variance
APC	Antigen-presentation cell
APRIL	A proliferation-inducing ligand
BAFF	B-cell activating factor
BAL	Bronchoalveolar Lavage
BCR	B-cell Receptor
BM	Bone Marrow
Breg	Regulatory B-cells
CA	Cancer control
CMI	Cell-mediated Immunity
COPD	Chronic Obstructive Pulmonary Disease
Ct	Cycle threshold
CTL	Cytotoxic Lymphocyte
Ctrl	Control
DC	Dendritic cell
DMSO	Dimethyl Sulfoxide
Dx	Diagnosis
EAE	Experimental autoimmune encephalomyelitis
EOT	End of treatment
FACS	Fluorescence-activated cell sorter
FasL	Fas Ligand
FCGR1A	Fc Gamma Receptor 1 A
FCS	Fetal Calf Serum
FO	Follicular
GC	Germinal Centre
GM-CSF	Granulocyte macrophage colony stimulating factor

HIV	Human immunodeficient virus
IFNγ	Interferon gamma
IL	Interleukin
IL5RA	Interleukin 5 Receptor Alpha
LTBI	Latent Tuberculosis Infection
M.tb	*Mycobacterium tuberculosis*
MHC	Major Histocompatibility Complex
MZ	Marginal Zone
NK	Natural Killer
OLD	Other Lung disease
PAMP	Pathogen-associated molecular patterns
PBMC	Peripheral Blood Mononuclear Cell
PBS	Phosphate Buffer Saline
PC	Plasma cell
PD1	Programmed Death Receptor 1
PRR	Pattern Recognition Receptors
QFN	QuantiFERON
RT-qPCR	Real-time quantitative Polymerase Chain Reaction
sFasL	soluble Fas Ligand
TB	Tuberculosis
TCR	T-cell Receptor
TD	Thymus-Dependent
TGF-β	Transforming Growth Factor-beta
Th	T helper
TI	Thymus-Independent
TLR	Toll-like Receptor
TNFα	Tumour Necrosis Factor alpha
TR	Transitional

Table 1. Cell Phenotypes

Cell type	Phenotype	Function
"Killer" B-cells	$CD19^{+}IgM^{+}CD38^{+}FASL^{+}IL5RA^{+}$	Apoptosis of CD4+ T-cells?
Regulatory B-cells (Breg)	CD19+CD24hiCD38hi	Regulate inflammatory responses
Memory B-cells (Bmemory)	CD19+CD24hiCD38-	Rapid secondary antibody response
Effector T-cells (TE)	CD3+CD4+CD25+	T-cells that carry out cell-mediated responses
Regulatory T-cells (Treg)	CD3+CD4+CD25hi	Immunomodulatory

Chapter 1. Introduction and Literature review

1.1 Immunology of Tuberculosis

Tuberculosis disease is caused by *Mycobacterium tuberculosis* (*M.tb*). The bacteria is spread by means of coughing, and the inhalation of sputum droplets which contain the bacteria. Once the bacteria are in the airways and lung parenchyma, macrophages and neutrophils are the first cells at the site of infection, which then phagocytose and take up the bacilli. The phagocytes (i.e. macrophages) recognize pathogen-associated molecular patterns (PAMPs) through toll-like receptors which are pattern recognition receptors (PRRs) [1]. This is considered the innate immune response. The natural killer (NK) cells migrate to the site of infection where they lyse the bacteria directly, as well as the infected macrophages [2], [3]. When neutrophils and macrophages undergo apoptosis, the bacilli are released in a contained manner and are taken up by dendritic cells (DC). These DC's then travel to lymph nodes, where it produces interleukin (IL)-12 which induces naïve T-cells to become T-helper (Th) 1 cells. The Th1 cells subsequently traffic to the lung, where it produces amongst other cytokines, interferon (IFN)-gamma and activates macrophages which leads to bacterial control and cytokine production [4]. Mouse studies have shown that the production of IFNγ leads to the production of iNOS, which is required to control *M.tb* infection [5], [6]. The interaction with antigen-presenting cells (APC), such as B-cells and DCs leads to the adaptive immune response, which comprises of humoral and cell-mediated immunity (CMI). B-cells, once activated, induces the humoral immune response by primarily producing antibody which assists in fighting invading pathogens. The CMI is essential in the control of *M.tb* infection because it leads to the production of essential pro-inflammatory cytokines such as IFNγ, IL2 and tumour necrosis factor (TNF)-α. CMI is initiated when T-cells interact with APCs, which leads to activation and production of cytokines and growth factors. IFNγ production leads to the recruitment of $CD4^+$ and $CD8^+$ T-cells to the site of infection, and

also activates macrophages to kill the bacteria [7]. TNFα is involved in cell migration, as it influences the expression of chemokines and chemokine receptors [8]. Therefore TNFα plays a role in granuloma formation, which is the aggregation of lymphocytes and macrophages in infected tissues in order to contain the bacterial infection. IL2 induces proliferation and expansion of antigen-specific lymphocytes [8]. It has been shown that $CD4^+$ Th1 cells are important for protection during *M.tb* infection, and that multi-functional $CD4^+$ T-cells, which produces INFγ, TNFα and IL2, correlate with TB disease [9]. Caccamo and colleagues (2010) showed that multifunctional $CD4^+$ Th1 cells are detected in 85 to 90% of TB cases, and the frequency of these multifunctional T-cells decline after anti-TB treatment [9]. However, the relevance of these multifunctional T-cells present during latency or induced by *M.tb* during TB disease remains unclear [10], [11].

1.2. T-cell activation

T-cells are activated by ligation of the T-cell receptor (TCR) by antigenic peptides in the context of Major Histocompatibility Complex (MHC). The TCR may contain either αβ chains (majority), which is found on mature T-cells, or the TCR may contain γδ chains [12]. The αβTCR is associated with co-receptor molecules, such as CD4 and CD8, which determines the effector functions of the T-cells subsequent to activation. These co-receptor molecules also determine which cells will present antigenic peptides to the T-cells. More specifically, $CD4^+$ T-cells will recognise antigen presented via MHC II, which is expressed by all professional APCs such as DCs, B cells and macrophages. Once the TCR of $CD4^+$ T-cells have been engaged, it results in signalling pathways that lead to the activation of these cells to become effector T helper (Th) cells [13]. T-cells mostly require 2 signals to become activated, one through the TCR and the second

through the interaction/binding of co-stimulatory molecules. These Th cells produce various cytokines, depending on the antigen and the microenvironment driving the lineage of Th cells differentiation. For instance, Th1 cells are induced by IL12 and IFNγ, and in an autocrine fashion produce IFNγ, IL2 and TNFα. These cytokines induce pro-inflammatory cell-mediated immunity, and also induce the production of opsonizing antibodies by B-cells, which results in the killing of invading pathogens. In contrast, Th2 cells are induced by IL4, and produce IL5, IL6, IL4 and IL13 [14]. These cytokines have an anti-inflammatory effect. In addition to the Th1 and Th2 lineages, T-cells may also become Th17 and T-regulatory (Treg) cells, where these cells produce IL17 and IL10, respectively. $CD8^+$ T-cells recognise antigen presented by MHC I, which is expressed on all nucleated cells. Once activated, $CD8^+$ T-cells will form effector cytotoxic T lymphocytes (CTLs), which induce killing of infected cells [15]. This killing activity is induced mainly by the release of Granzyme-A and –B, and perforin. T-cells require co-stimulation for activation, and in the same way provide co-stimulation to APCs, such as B-cells, as well.

1.3. B-cell activation and effector functions

B-cells are well known for the production of antibodies which is part of the humoral immune responses. B-cells develop from a progenitor in the bone marrow (BM), and enter the periphery once it has gone through several developmental stages and forms immature B-cells. B-cells split into two major lineages, B1 and B2. The B1 lineage is much less understood, whilst the B2 lineage can be further subdivided into follicular (FO), marginal zone (MZ) and transitional (TR), germinal centre (GC) and memory B (MB) -cells. The immature cells leave the BM as TR cells, after which the B-cells can enter either the FO or MZ subset [16]. Marginal zone cells home to the MZ in the splenic white pulp, whereas FO cells recirculate in blood and secondary lymphoid organs. In order

for B-cells to be activated it requires two signals, similar to the activation of T-cells. The first signal is provided when the B-cell receptor (BCR) is ligated by antigen. The second signal may be provided by T-cells (Thymus-dependent (TD)) or through toll-like receptors or via intense BCR cross linking (Thymus-independent (TI)) [16]. During TD responses, germinal centres form in the lymph nodes and spleen where proliferating B-cells appear at the margins of T-cell zones and B-cell follicles. This is where plasma cells (PCs) and MBs arise. PCs produce antibodies, which assists in the defence against invading pathogens, but is not sufficient for the control of infection[17].

In addition to the production of antibodies, B-cells are also APCs and studies show that effector B-cells and plasma B-cells are also able to produce cytokines [18], [19]. Similar to T-cells, the cytokines produced depends on the lineage of cytokine-producing B-cells. In addition, the cytokines produced by the B-cells may depend on the local cytokine environment. It has been proposed that if the micro-environment contains Th1 cytokines, such as IFNγ and IL2, B-cells will form B effector (Be)-1 cells and will produce the Th1-like cytokines. Similarly, if the micro-environment contains Th2 cytokines, such as IL5 and IL4, Be-2 cells will be induced. B-cells can differentiate into regulatory B-cells (Bregs) as well [20]–[22]. These B-cells produce cytokines like TGF-β and IL10, and express death inducing ligands like FasL[23]. Furthermore, $CD19^{+}CD24^{hi}CD38^{hi}$ Bregs produce cytokine such as IL10 and suppress effector T-cell function [24]. A study by Wang and colleagues (2015) indicate that Bregs induce Tregs during gastric cancer, and suppress effector T-cells (TEs) (Figure 1.1) [25]. Furthermore, literature shows that this suppression of TEs leads to insufficient bacterial clearance [26], [27]. Prior work from our group indicated that B-cells with a plasmablast phenotype ($CD138^{+}$) produced the majority of cytokines secreted in response to stimulation with BCG and TLR9-agonist [28]. Similarly, a study

on parasites found that IL17-producing plasmablasts played an important role in combating disease pathology [29].

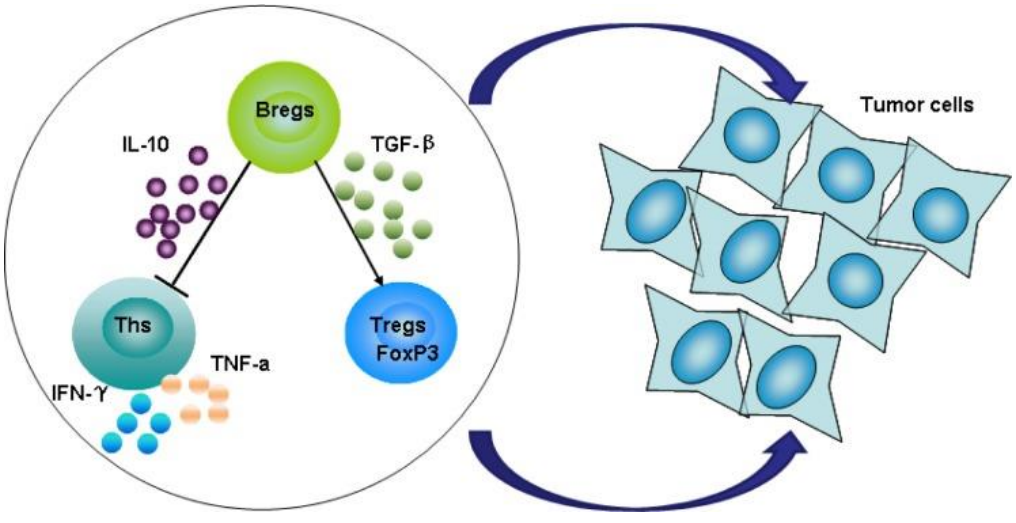

Figure 1.1. Effects of Bregs on Tregs and Effector T-cells during gastric cancer
The frequency of regulatory B-cells is increased during gastric cancer, which induces Tregs and suppresses effector T-cell function by means of IL10 production. These Treg cells, in turn, have a suppressive effect on tumour cells [25].

1.4. Relationship between B- and T-cells

As mentioned above, in order for T-cells to become activated, two signals are required. The first signal is provided by the binding of MHC/peptide complex to the TCR and CD4/CD8 co-receptor. The second co-stimulatory signal, is provided by the binding of CD80 or CD86, which is expressed on the APC to CD28 expressed on the T-cells [12]. In the case of B-cells acting as APC, MHC II/peptide complex binds to the TCR and CD4 co-receptor. In addition to this, T-cells provide a co-stimulatory signal to the APC (B-cells) by the binding of CD40L expressed by T-cells to CD40 expressed by APC (B-cells). This co-stimulation increase the expression of MHC by the APCs, as well as co-stimulatory receptors. However, what remains unclear is the extent of the relationship between B- and T-cells during immune activation and infection with *M.tb*.

Due to the additional effector functions of B-cells, namely APC and the production of cytokines, B-cells have the potential to modulate T-cell behaviour. Cytokines produced by B-cells may induce different effector T-cells (Figure 1.2). For instance, Be-1 cells may have the potential to induce Th1 cells. Similarly, if Be cells produce Th2-like cytokines (Be-2 cells), it may induce naïve T-cells to become Th2 cells. This is an important hypothesis to explore due to the significant role assigned to T-cells during TB. In addition to the production of cytokine and presenting antigen to T-cells, B-cells have the potential to induce cell death by expressing death ligands [23]. Studies show that a small subset of regulatory B-cells express Fas-Ligand (FasL) [23]. FasL (CD178) induces cell death once it binds to Fas (CD95) on the surface of target cells. Evidence show that B-cells expressing FasL has the potential to induce apoptosis of $CD4^+$ T-cells [30]. These studies were focussed on autoimmunity, however evidence exists that indicate that B-cells may behave similarly during TB disease, where this phenotype was shown to be induced when stimulated with bacterial LPS and CpG[1], [31]–[34].

1.5. Hypothesis

Considering the current literature, we hypothesize that B-cells utilise alternative effector functions during TB disease which may play an essential role in the host defence against infection, such as the production of cytokine or the expression of regulatory factors. Furthermore, we hypothesize that B-cells play an important role in the modulation of T-cell responses to invading pathogens.

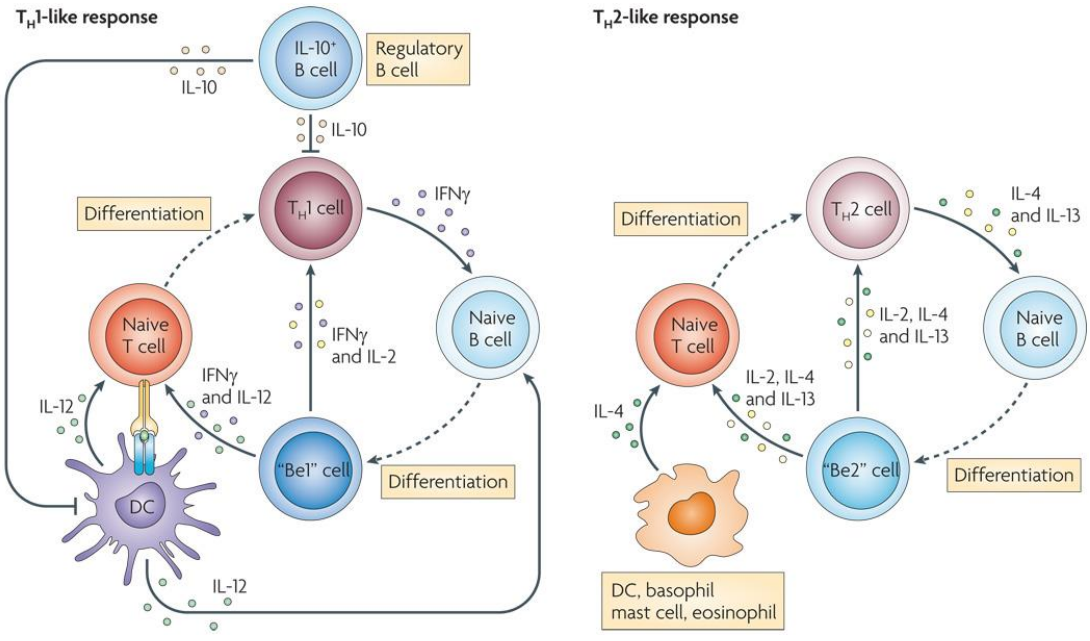

Figure 1.2. Proposed relationship between cytokine-producing B-cells and T-cells
Cytokines produced by Be1 cells (IFNγ) induce naïve T-cells to differentiate into Th1 cells, which then produce cytokine that maintain Be1 cells. Similarly, Be2 cells produce cytokine (IL4) that induce naïve T-cells to differentiate into Th2 cells. These cells subsequently produce cytokines that maintain Be2 cells [24].

1.6 Aims and Objectives

With this study we aimed to evaluate the B-cell behaviour during *M.tb* infection, and how anti-TB treatment affects B-cell non-humoral responses. We also investigated how B-cells modulate T-cell profiles and subsequent responses.

The first objective was to evaluate the B-cell gene regulation during *M.tb* infection and TB treatment by means of transcriptomic analyses (Chapter 2). Secondly, by means of flow cytometry we characterised the phenotype of B-cells present during TB disease and how these cells are affected during anti-TB treatment (Chapter 3). Finally, we were interested in how B-cells shape the T-cell phenotype following co-culture under varying conditions to elucidate the role of B-cells during T-cell modulation (Chapter 4).

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Successful TB treatment induces B-cells expressing FASL and IL5RA mRNA

Ilana C. van Rensburg[1], Chandre Wagman[1], Kim Stanley[1], Caroline Beltran[1], Katharina Ronacher[1], Gerhard Walzl[1] and Andre G. Loxton[1]

[1] SA MRC Centre for TB Research, DST/NRF Centre of Excellence for Biomedical Tuberculosis Research, Division of Molecular Biology and Human Genetics, Faculty of Medicine and Health Sciences, Stellenbosch University, Cape Town, South Africa

Correspondence to: Andre G. Loxton, *email:* GL2@sun.ac.za

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ABSTRACT

Activated B-cells increase T-cell behaviour during autoimmune disease and other infections by means of cytokine production and antigen-presentation. Functional studies in experimental autoimmune encephalomyelitis (EAE) indicate that B-cell deficiencies, and a lack of *IL10* and *IL35* leads to a poor prognosis. We hypothesised that B-cells play a role during tuberculosis. We evaluated B-cell mRNA expression using real-time PCR from healthy community controls, individuals with other lung diseases and newly diagnosed untreated pulmonary TB patients at three different time points (diagnosis, month 2 and 6 of treatment).

We show that *FASLG, IL5RA, CD38* and *IL4* expression was lower in B-cells from TB cases compared to healthy controls. The changes in expression levels of *CD38* may be due to a reduced activation of B-cells from TB cases at diagnosis. By month 2 of treatment, there was a significant increase in the expression of *APRIL* and *IL5RA* in TB cases. Furthermore, after 6 months of treatment, *APRIL, FASLG, IL5RA and CD19* were upregulated in B-cells from TB cases. The increase in the expression of *APRIL* and *CD19* suggests that there may be restored activation of B-cells following anti-TB treatment. The upregulation of *FASLG* and *IL5RA* indicates that B-cells expressing regulatory genes may play an important role in the protective immunity against *M.tb* infection. Our results show that increased activation of B-cells is present following successful TB treatment, and that the expression of *FASLG* and *IL5RA* could potentially be utilised as a signature to monitor treatment response.

Chapter 2. Successful TB treatment induces B-cells expressing FASL and IL5RA mRNA

2.1. Abstract

Activated B-cells increase T-cell behaviour during autoimmune disease and other infections by means of cytokine production and antigen-presentation. Functional studies in experimental autoimmune encephalomyelitis (EAE) indicate that B-cell deficiencies, and a lack of *IL10* and *IL35* leads to a poor prognosis. We hypothesised that B-cells play a role during tuberculosis. We evaluated B-cell mRNA expression using real-time PCR from healthy community controls, individuals with other lung diseases and newly diagnosed untreated pulmonary TB patients at three different time points (diagnosis, month 2 and 6 of treatment).

We show that *FASLG, IL5RA, CD38* and *IL4* expression was lower in B-cells from TB cases compared to healthy controls. The changes in expression levels of *CD38* may be due to a reduced activation of B-cells from TB cases at diagnosis. By month 2 of treatment, there was a significant increase in the expression of *APRIL* and *IL5RA* in TB cases. Furthermore, after 6 months of treatment, *APRIL, FASLG, IL5RA and CD19* were upregulated in B-cells from TB cases. The increase in the expression of *APRIL* and *CD19* suggests that there may be restored activation of B-cells following anti-TB treatment. The upregulation of *FASLG* and *IL5RA* indicates that B-cells expressing regulatory genes may play an important role in the protective immunity against *M.tb* infection. Our results show that increased activation of B-cells is present following successful TB treatment, and that the expression of *FASLG* and *IL5RA* could potentially be utilised as a signature to monitor treatment response.

2.2. Introduction

T-cell driven cell-mediated immunity, have been the focus of studies investigating host immunity against *M.tb* infection [35]–[37] and TB disease. However, an increasing number of studies are indicating that the role of B-cells in the protective immunity against *M.tb* infection has been underestimated. B-cells are the key players during humoral immunity and produce antibodies in response to invading pathogens. Studies found that humoral immunity may enhance protection against *M.tb* infection [38]–[40]. Furthermore, B-cells have additional functions that may be essential for host protection during infection and disease. Activated B-cells change T-cell behaviour during autoimmune disease and other pathogenic infections by means of cytokine production and antigen-presentation. Effector B-cells can either produce IFNγ and IL12 (Be-1 cells) or IL2, IL13 and IL4 (Be-2 cells) depending on whether the cells are primed by Th1 or Th2 cells, respectively [41], [42]. These cytokine-producing effector B-cells are able to amplify T-cell responses in a cytokine-dependent manner [43], [44].

B-cells also display regulatory phenotypes, which secrete IL10, IL35, and express FAS ligand (FasL). Functional studies in Experimental autoimmune encephalomyelitis (EAE) indicate that B-cell deficiencies, and a lack of *IL10* and *IL35* lead to a poor prognosis[45]. B-cells limited EAE pathogenesis by means of IL35 secretion, which decreased the accumulation of pathogenic cells in the target organ. FasL-expressing B-cells have a similar effect during autoimmune disease. These B-cells induce apoptosis of $CD4^+$ T-cells, have the potential to reduce the inflammatory responses during autoimmune disease and are induced by IL5 [46]. High levels of FasL expression was observed in B-cells activated via the Toll-Like Receptor (TLR) 9 agonist CpG [47]. We hypothesised that B-cells play a similar role during tuberculosis disease. Transcriptional approaches are useful for the discovery of biomarkers for the diagnosis and measurement of treatment response [48]–[51]. We evaluated patterns in the expression of B-cell genes to better

our understanding of B-cell behaviour during *M.tb* infection and tuberculosis (TB). Real-time PCR was utilised to assess the expression of gene transcripts of cytokines, together with genes involved in B-cell activation and effector functions.

2.3. Materials and Methods

2.3.1. Participant recruitment and Sample collection

This study was carried out according to the Helsinki Declaration and International Conference of Harmonisation guidelines. All participants provided written informed consent for participation in the study, and the use and storage of the samples for biomarker discovery. Ethical approval was obtained from the Health Research Ethics Committee (Ethics number N10/01/013) of Stellenbosch University and the Departments of Health of the Province of the Western Cape and City of Cape Town.

The study participants were recruited from the Ravensmead/Uitsig community in Cape Town Western Cape. Individuals with newly diagnosed, untreated pulmonary TB were enrolled into the study prior to commencement of anti-TB treatment. The inclusion criteria for the ten healthy controls was a positive QuantiFERON (QFN) test. For TB cases we included participants with a chest radiograph with suggestive signs of TB, clinical symptoms of TB including a chronic cough, weight loss and night sweats, as well as a positive sputum-culture and -smear. The exclusion criteria included prior cases of TB or other lung diseases, and an HIV positive status. In addition, ten individuals with a lung disease (other than TB) were enrolled into the study. Half of the other lung disease (OLDs) (Table 2.1) were QFN positive. The individuals in the OLD and healthy control (Ctrl) groups did not present any clinical symptoms of *M.tb* infection.

Peripheral blood was collected from the study participants into sodium heparin tubes, as well as sputum samples for smear/culture tests. The individuals with TB were subsequently followed up at month two and month six of treatment, where blood and sputum samples were collected once more to monitor their response to treatment. Thirteen TB cases had converted to culture negative at month 2 of treatment, and all TB cases were cured at the end of treatment which was confirmed by sputum culture tests.

Table 2.1. Clinical and demographic characteristics of study participants

	TB	CTRL	OLD
No. of Female	8	10	5
No. of Male	12	0	5
QuantiFERON status (Dx)	NA	POSITIVE	5 POSITIVE
			5 NEGATIVE
Sputum-culture status (Dx)	POSITIVE	NEGATIVE	NEGATIVE

2.3.2. Sample preparation, mRNA isolation and Quality Check

On the day of blood collection, peripheral blood mononuclear cells (PBMCs) were isolated using the ficoll/histopaque separation method (GE Health, Piscataway, NJ). Total B-cells were isolated using the MACS bead separation method through positive selection (B cell isolation kit II, Miltenyi, Germany), and stored in RNAlater® (Life Technologies, USA). The cells were stored in liquid nitrogen until batch analysis.

The cells were thawed on ice and spun down before being washed in Phosphate-buffered saline (PBS). Subsequently, mRNA was isolated using the RNEasy® Mini Kit (Qiagen, Germany)

according to manufacturer's instructions. RNA purity and quantification was assessed using NanoDrop 2000 spectrophotometer data. More specifically, the 260/ 280 ratio and the 260/ 230 ratio were measured to assess the purity of the isolated RNA. A 260/280 and 260/230 ratio of at least 1.7 and 1.5 respectively, was considered sufficiently pure for further analysis. The RNA samples were stored at -80 ˚C.

2.3.3. cDNA synthesis and RT-qPCR

The isolated RNA was thawed on ice and diluted to a concentration of 300ug, and subsequently used to synthesise cDNA. The procedure was carried out in a thermal cycler (Life Technologies, USA) using the First Strand Kit (Qiagen, Germany) according to the manufacturer's instructions. The cDNA was then used as template for quantitative PCR on the ABI 7900HT platform. The RT^2 Profiler Custom Arrays (Qiagen, Germany) were utilised and manufacturer's instructions were followed. The arrays contained primers for the following genes of interest: *IL35* (NM_000882.3), *IL4*(NM_000589.3), Fas-ligand (*FASLG*) (NM000639.2), *IL5RA* receptor alpha (*IL5RA*) (NM_000564.4), cluster of differentiation 38 (*CD38*) (NM_001775.2), *APRIL* (NM_00198622.1), *BAFF* (NM001145645.2), *TNF-a* (NM_000594.3), Fc gamma receptor 1 alpha (*FCGR1A)* (NM_000566.3), Toll-like receptor 9 (*TLR9*) (NM_017442.3), *STAT6* (NM_001178078.1), as well as two housekeeping genes *B2M* (NM_004048) and *GAPDH* (NM_002046). *FASLG, IL5RA* and *IL35* is associated with regulatory B-cells. Furthermore, *APRIL, CD38* and *TLR9* is involved in B-cells activation.

2.3.4. Data Analysis

The gene expression data obtained from the ABI 7900HT was represented as Ct values, which indicated the earliest visible cycle of amplification. These Ct values were converted to fold change values using the Qiagen online software (www.SABiosciences.com/pcrarraydataanalysis.php). Where applicable, differences were calculated using Prism 5 Software and Kruskal-Wallis tests, where $p < 0.05$ indicates a statistically significant difference. Heatmaps were generated using delta Ct (dCt) data and R statistical packages.

2.4. Results

2.4.1. Comparison of gene expression between TB, OLD and Control groups

In order to evaluate the differences in the expression of the 12 B-cell specific genes between healthy controls (Ctrl), TB cases and individuals with other lung diseases (OLD), the delta Ct method was utilised. All analyses were carried out by means of the online software provided by SABiosciences.

The expression of *APRIL, BAFF, CD19, IL4, FCGR1A, IL35, IL5RA, TNF and TLR9* was similar between Ctrl and OLD groups ($p>0.05$, Table 2.2). In contrast, *FASLG* and *CD38* expression was significantly decreased in OLD compared to Ctrl ($p<0.01$; Table 2.2). There were no differences in the expression of all the genes between individuals with other lung diseases and TB cases (Table 2.2). When comparing the expression of the genes between healthy controls and TB cases, we found that there was a significant decrease in the expression of *IL4, FASLG, IL5RA* and *CD38* ($p<0.05$) (Table 2.2). The relative gene expression changes between the CTRL, OLD and TB

groups are shown in in the form of a heat map (Figure 2.1), which demonstrates differential expression of genes between the control group and the TB group is evident.

Table 2.2. Gene expression differences between TB and Control Groups
The fold change values represent the changes in gene expression between the respective groups, as calculated by SABiosciences online software. Positive values indicate an increase in expression, (negative value indicates a decrease in expression) between groups. Kruskal-Wallis tests were used for comparisons, and a p-value < 0.05 is considered significant and is highlighted in bold.

GENES	Ctrl vs OLD		Ctrl vs TB		OLD vs TB	
	Fold Change	**p-value**	**Fold Change**	**p-value**	**Fold Change**	**p-value**
APRIL	-2.08	p > 0.05	-3.03	p > 0.05	-1.46	p > 0.05
TLR9	-1.60	p > 0.05	-2.10	p > 0.05	-1.31	p > 0.05
IL5RA	-3.54	p > 0.05	**-8.26**	**p < 0.01**	-2.33	p > 0.05
IL4	-7.03	p > 0.05	**-10.70**	**p < 0.01**	-1.52	p > 0.05
IL35	-2.77	p > 0.05	-3.35	p > 0.05	-1.21	p > 0.05
FASLG	**-6.26**	**p < 0.01**	**-5.70**	**p < 0.01**	1.10	p > 0.05
FCGR1A	-1.78	p > 0.05	1.39	p > 0.05	2.47	p > 0.05
BAFF	-1.71	p > 0.05	-1.74	p > 0.05	-1.02	p > 0.05
TNF	**1.63**	**p < 0.05**	1.12	p > 0.05	-1.45	p > 0.05
CD38	**-6.06**	**p < 0.01**	**-6.59**	**p < 0.01**	-1.09	p > 0.05
CD19	1.08	p > 0.05	-1.03	p > 0.05	-1.12	p > 0.05
STAT6	-1.57	p > 0.05	-1.66	p > 0.05	-1.06	p > 0.05

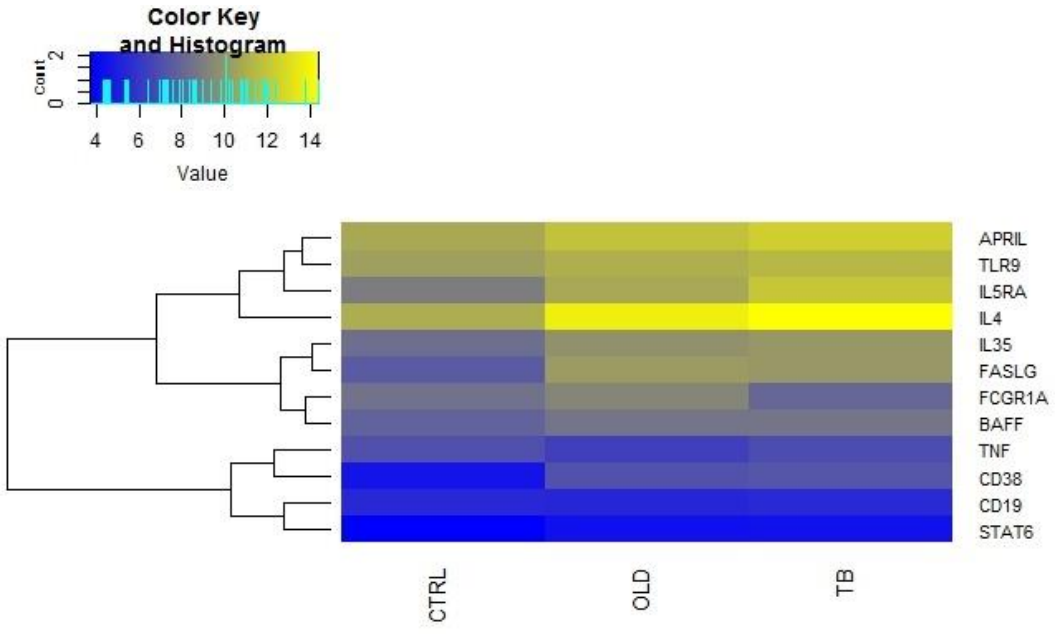


Figure 2.1. Differences in mRNA expression between TB and Control groups
Comparison of the average gene expression between the respective groups. Where Ctrl are healthy controls, OLD are individuals with other lung diseases and TB are individuals is TB disease. Heatmap, which was generated using R statistical packages, depicting changes in relative gene expression (dCt). Genes with low expression levels are depicted in yellow, whereas genes with high expression levels are depicted in blue.

2.4.2. Differential gene expression during Anti-TB treatment

The expression of the B-cell specific genes was compared between diagnosis (before treatment started), month two (the time point frequently used to evaluate sputum culture conversion) and month six (end of treatment) following the initiation of anti-TB treatment. There were no significant differences in the expression of *BAFF*, *CD19*, *FASLG*, *IL4*, *CD38*, *FCGR1A*, *IL35*, *TNF* and *TLR9* between diagnosis and month 2 of treatment ($p>0.05$) (Table 2.3). However, *APRIL* and *IL5RA* were differentially expressed between these two time points with fold changes of 1.85 ($p<0.05$) and 1.87 ($p<0.05$) respectively (Table 2.3).

Table 2.3. Gene expression in TB group over 6 months of treatment
The fold change values represent the changes in gene expression between the respective groups, as calculated by SABiosciences online software. Positive values indicate an increase in expression, whereas a negative value indicates a decrease in expression. Kruskal Wallis tests were used for comparisons, and a p-value ≤ 0.05 is considered significant and is highlighted in bold.

GENES	Dx vs M2		Dx vs M6		M2 vs M6	
	Fold Change	**p-value**	**Fold Change**	**p-value**	**Fold Change**	**p-value**
APRIL	**1.85**	**p < 0.05**	**2.06**	**p < 0.01**	**1.12**	p > 0.05
IL5RA	**1.87**	**p < 0.05**	**1.96**	**p < 0.05**	**1.05**	p > 0.05
TLR9	1.18	p > 0.05	1.35	p > 0.05	1.15	p > 0.05
IL4	1.79	p > 0.05	1.84	p > 0.05	1.03	p > 0.05
IL35	1.04	p > 0.05	1.21	p > 0.05	1.17	p > 0.05
FASLG	1.33	p > 0.05	**1.87**	**p < 0.01**	1.40	p > 0.05
FCGR1A	-1.91	p > 0.05	-2.74	p > 0.05	-1.43	p > 0.05
BAFF	1.13	p > 0.05	1.00	p > 0.05	-1.12	p > 0.05
TNF	1.10	p > 0.05	-1.02	p > 0.05	-1.12	p > 0.05
CD38	1.65	p > 0.05	1.48	p > 0.05	1.11	p > 0.05
CD19	1.49	p > 0.05	**1.77**	**p < 0.05**	1.18	p > 0.05
STAT6	1.08	p > 0.05	1.21	p > 0.05	1.12	p > 0.05

When comparing the expression of the 12 genes between diagnosis and month six of treatment, *APRIL, CD19, FASL and IL5RA* were differentially expressed (Table 2.3 and Figure 2.2). There was an increase in the expression of all the latter genes at month 6. The expression of *BAFF, STAT6, IL35, TNF, CD38* and *TLR9* was similar at diagnosis and month six of treatment. The relative gene expression is depicted in a heat map (Figure 2.3)

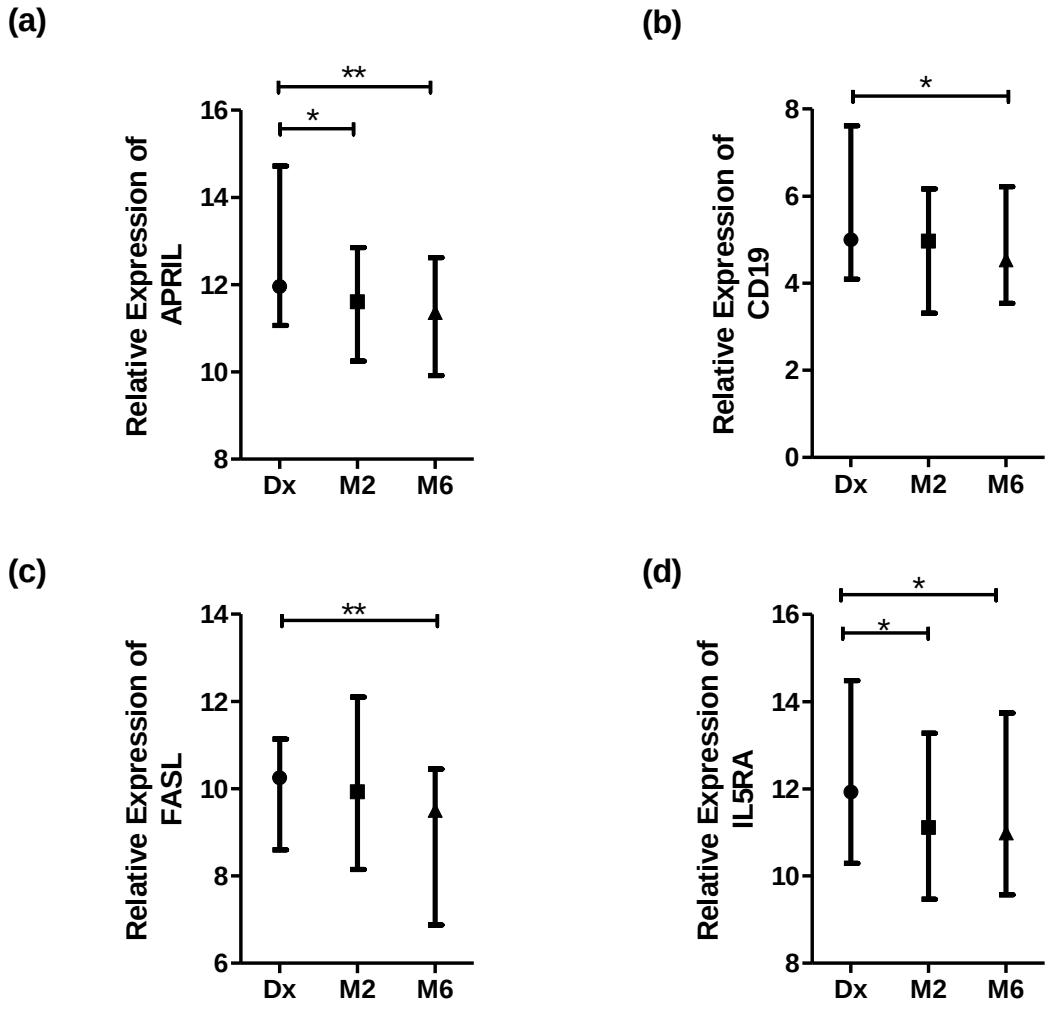
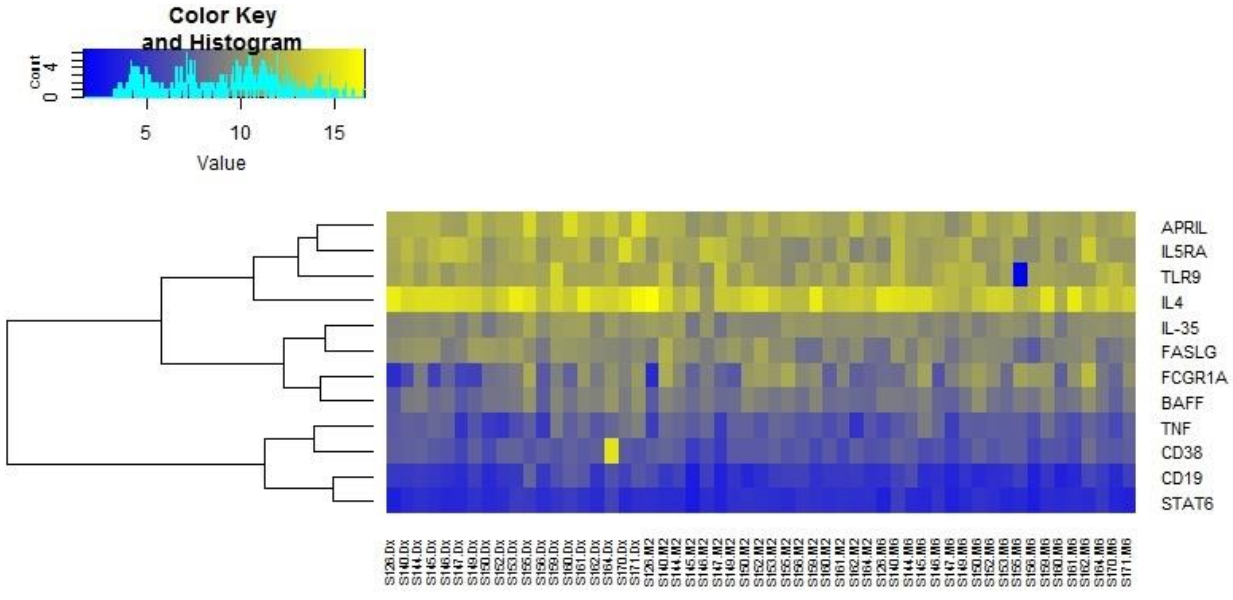


Figure 2.2. Genes differentially expressed during anti-TB treatment
Relative expression of genes as calculated using the dCt method (Ct (gene) – Ct (housekeeping gene)). Statistical differences calculated by means of Kruskal Wallis tests, where significant differences are indicated by an asterisk (* = p <0, 05 or ** = p<0.01). Data represented as median dCt with bars representing range. High dCt values indicate low gene expression, and low dCt values indicate high gene expression. (a) APRIL, (b) CD19, (c) FASL and (d) IL5RA.

(a)



(b)

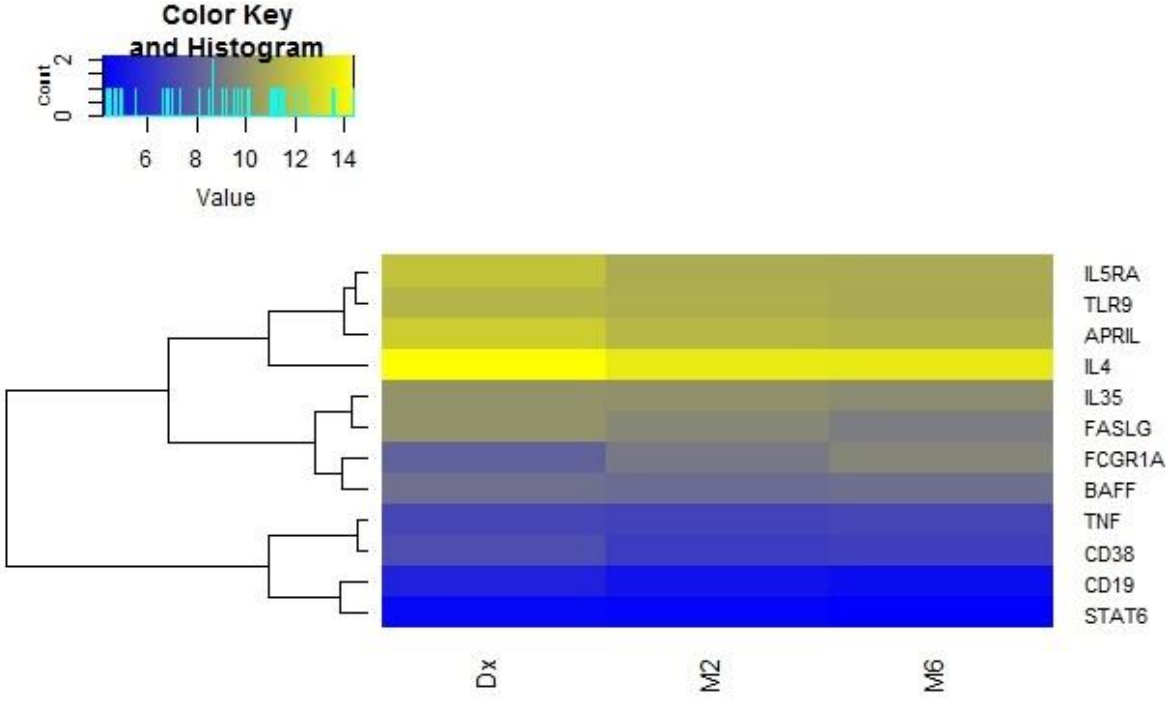


Figure 2.3. Differential mRNA expression over the course of anti-TB treatment
Heat map depicting changes in relative gene expression (dCt). Genes with low expression levels are depicted in yellow, whereas genes with high expression levels are depicted in blue. **(a)** Comparison of the average gene expression between the respective groups. **(b)** Group comparisons showing gene expression for individual participants.

2.5. Discussion

B-cells displaying regulatory phenotypes secrete cytokines such as IL10 and IL35, and also express FasL. These FasL-expressing B-cells have predominantly been studied in autoimmune diseases and infections [52]. A study conducted in mice showed that IL35 secreted by B-cells limited EAE pathogenesis by decreasing the accumulation of pathogenic cells in the target organ [45]. Similarly, B-cell-deficiencies in mice have been linked to a decreased control of *Salmonella* infection [53]. In the current study, *FASLG*, *IL5RA* and *IL4* expression were decreased in TB cases at diagnosis compared to healthy controls. In contrast, *FASLG* and *IL5RA* expression were increased after 6 months in TB cases. These results suggest that B-cell activity may be restored following successful anti-TB treatment and could play a role in the protective immunity against *M.tb* infection.

CD38 is a membrane-bound protein which is expressed on the surface of B-cells throughout their lifespan [54]. However, the expression of CD38 is highest on B1 B-cells [55]. Crosslinking of the CD38 receptor on mature B-cells leads to proliferation, isotype switching and reduced apoptosis. Additionally, CD38 acts as a co-stimulatory signal to B-cells activated by means of TLR ligands [56], [57]. Here we show a decline in the expression of *CD38* in B-cells from TB participants compared to healthy controls. This points to a reduction in the activation of B-cells during active *M.tb* infection. APRIL, which is a proliferation inducing ligand, is also associated with the activation of B-cells. We found *APRIL* at lower levels in TB participants compared to healthy controls. However, the expression of this gene was restored after 6 months of treatment, in addition to *CD19*. Together these results suggest that there is an increase in B-cell activation throughout treatment in TB cases. This restored B-cell activation may lead to more interactions between B-cells and helper T-cells due to the upregulation of co-stimulatory molecules [57].

Transcriptional analyses are useful for the identification of biomarkers for diagnostics, as well as monitoring treatment response during tuberculosis and other diseases [48]–[51]. In the current study, *FASLG* and *IL5RA* was downregulated in TB cases at diagnosis compared to healthy controls. The expression of the latter genes were upregulated in TB cases at 6 months of treatment. Collectively, this suggests that *FASLG* and *IL5RA* have the potential to be utilised as a biosignature to monitor success or failure of treatment. Further studies in larger cohorts are required to confirm this.

In conclusion, the results of our pilot study suggest that B-cells expressing regulatory genes could be involved during the immune response to *M.tb* infection. Previous studies of regulatory B-cells focussed mostly on autoimmune diseases and helminth infections. Supplementary transcriptional and functional studies are required to confirm the precise role that B cells play during tuberculosis disease and the mechanisms by which the activation/immune induction is achieved.

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Conflict of Interest

The authors declare no conflict of interest

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Contribution and Role in the study: I performed all the lab work, analysed the data and drafted the manuscript.

Chapter 3. B-cells with a FasL expressing regulatory phenotype are induced following successful anti-Tuberculosis treatment

3.1. Abstract

Studies show that B-cells, in addition to producing antibodies and antigen-presentation, are able to produce cytokines as well. These include regulatory cytokines such as IL-10 by regulatory B-cells. Furthermore, a rare regulatory subset of B-cells have the potential to express FasL, which is a death-inducing ligand. This subset of B-cells have a positive role during autoimmune disease, but has not yet been studied during tuberculosis. These FasL-expressing B-cells are induced by bacterial LPS and CpG, thus we hypothesised that this phenotype might be induced during tuberculosis as well.

B-cells from participants with TB (at diagnosis and during treatment) and controls were collected, and analysed by means of real-time PCR and flow cytometry. In addition to this, BAL was collected from TB participants as well and analysed by means of MAGPix (multi-cytokine) technology.

Gene expression analysis show that FASL transcript levels increase by the end of treatment. Similarly, phenotypic analysis show that there is a higher frequency of FasL-expressing B-cells by the end of treatment.

Collectively, these results indicate that these FasL-expressing B-cells are being induced during anti-TB treatment, and thus may play a positive role. Further studies are required to elucidate this.

3.2. Introduction

B lymphocytes (B-cells) are predominantly recognised for its role during humoral immunity, whereby they produce antibody to assist in fighting infections. However, B-cells are able to produce cytokine as well, and the type of cytokines produced is dependent on the microenvironment. Effector type 1 B-cells (Be-1) produce Th1 type cytokines such as IFNγ and TNFα, Be-2 cells produce Th2 type cytokines while regulatory B-cells produce IL10 [20]. Cytokine-producing B-cells play important roles in the regulation of immunity and have been extensively studied in the context of autoimmune disease [20], [58]. B-cell culture studies have shown that there is a balance in the proinflammatory and regulatory responses of B-cells [59]–[61]. In the context of autoimmune disease, and more specifically multiple sclerosis (MS), this balance is skewed toward a more proinflammatory response due to a reduction in the production of IL10 [58]. The balance is however restored upon treatment. An additional regulatory function utilised by B-cells is the expression of Fas-Ligand (FasL) upon activation [62], [63]. This subset of B-cells is rare and has been studied in the context of autoimmune disease as well as helminth infections [64]. These regulatory "killer" B-cells are $CD19^{+}IgM^{+}$ and induce apoptosis of CD4 T-cells, and thus have a positive effect during autoimmune disease. The B-cells induce FasL-mediated apoptosis of autoreactive T-cells, thereby regulating/reducing the inflammation. Klinker and colleagues (2013) showed that this regulatory subset of B-cells are induced by IL5, and that there is an increase in the expression of IL5 receptor alpha (IL5Rα) [64]. Lymphocytes may undergo exhaustion due to persistent infections and prolonged activation. This occurs as the cells fail to response to antigenic challenge, and has increased expression of markers of exhaustion such as programmed cell death protein (PD) 1. The role of these 'killer' B-cells during tuberculosis has not yet been studied, but it was shown that this phenotype is induced in B-cells upon stimulation with TLR9 agonist CpG [47]. This may imply that the same regulatory

phenotype could be induced in the context of TB, as B-cell TLR9 is triggered by mycobacterial CpG motifs [1], [31], [33], [34]. Thus, we hypothesised that FasL expression in B-cells is induced during *M.tb* infection. We therefore aimed to evaluate gene expression patterns of FASL and IL5RA by B-cells using real-time PCR. Additionally, we aimed to evaluate the phenotype of B-cells from individuals with TB by means of flow cytometry, and how this changes throughout treatment. Finally, we evaluated host marker levels in BAL and plasma from individuals with TB.

3.3. Materials and Methods

3.3.1. Ethics Statement

Ethical approval was obtained from the ethics committee of Stellenbosch University (N10/01/013 78 and N13/05/064) and the City of Cape Town City Health. The study was conducted according to the Helsinki Declaration and International Conference of Harmonisation guidelines. Written informed consent was obtained from all study participants.

3.3.2. Participant recruitment

All participants were recruited in Ravensmead/Uitsig Community in Cape Town. Newly diagnosed TB were recruited for the study before initiation of treatment. The inclusion criteria is shown in table 1. The TB cases were followed up during the course of 6 month treatment. In addition, healthy controls (CTRL) and individuals with other lung diseases (OLD) including pneumonia, asthma exacerbations, pleuritis and chronic obstructive pulmonary disease (COPD) were recruited. The healthy controls were all QuantiFERON (QFN) positive, which is indicative of latent TB infection (LTBI). Participants with cancer were included in the study as controls for the bronchoscopies (Addendum A). These participants displayed respiratory symptoms due to

inflammatory or infective cause, but were sputum-culture and Gene Xpert negative, which indicated that they did not have TB. Individuals who had recently completed TB treatment (within 3 months) and were sputum-culture negative were selected for bronchoscopies. Bronchoscopies were performed at Tygerberg Hospital, Cape Town, and transported to the laboratory under controlled conditions for processing.

Table 3.2. Inclusion and Exclusion criteria for participant recruitment

TB	CTRL	OLD
• Clinical signs of TB* • Chest radiographs with sign of TB • Positive sputum culture test • HIV negative	• No clinical signs of TB* • Chest radiographs with no signs of TB • Negative sputum culture test • QFN positive • HIV negative	• No clinical signs of TB* • Negative sputum tests • No prior or current TB • HIV negative

* cough, fever, night sweats, weight loss, loss of appetite

3.3.3. B-cell phenotype analysis using Flow cytometric analysis

One ml of sodium heparin blood was collected from 13 CTRLs (once-off) and 13 TB cases at diagnosis, week 2, month 1 and month 6 of treatment. The blood was lysed using 1x BD fluorescence-activated cell sorter (FACS) Lysing solution (BD, New Jersey, USA), and the leukocytes were cryopreserved in fetal calf serum (FCS, BIOCOM Biotech) containing 10% DMSO (Sigma, Missouri, US), placed in a Mr. Frosty at -80˚C overnight and transferred to liquid nitrogen the following day.

Once all the samples had been collected, cells were retrieved from liquid nitrogen and washed in FACS buffer (PBS containing 2% FCS). The cells were then stained for 30 minutes with the

following antibodies; CD19-BV510, IgM-FITC, CD125w (or IL5RA)-PE, CD3-PerCP, PD1-BV421, CD40-APC-H7, CD38-PE-Cy7 and CD178 (or FASL)-APC. Subsequently, the samples were analysed using the FACS Canto II (BD) and the data was analysed using FlowJo software (Version 10, Oregon, US).

3.3.4. B-cell gene expression analysis

Blood was collected into sodium heparin tubes from 10 CTRLs and 10 OLD (once-off), and 19 TB cases at diagnosis, month 2 and month 6 of treatment, and peripheral blood mononuclear cells (PBMCs) were isolated using the ficol-histopaque (Sigma-Aldrich, Missouri, US) separation method. Subsequently, total B-cells were isolated from the PBMCs by positive selection using the B-cell MACS Isolation kit (Miltenyi) according to the manufacturer's instructions. Cell purities were checked by flow cytometry and were above 90%.

RNA was isolated from these B-cells and used to synthesise cDNA. The procedure was carried out in a thermal cycler (Life Technologies, California, USA) using the First Strand Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Quantitative PCR was carried out using the ABI 7900HT platform (Applied Biosystems, California, US). The RT^2 Profiler Custom Arrays (Qiagen, Hilden, Germany) were utilised and manufacturer's instructions were followed. The arrays contained primers for Fas-ligand (*FASLG*) (NM000639.2) and *IL5RA* receptor alpha (*IL5RA*) (NM_000564.4) which are genes associated with regulatory FasL-expressing B-cells. The gene expression data obtained from the ABI 7900HT was represented as Ct values, which indicated the earliest visible cycle of amplification. These Ct values were converted to fold change values using the Qiagen online software (www.SABiosciences.com/pcrarraydataanalysis.php).

3.3.5. Host marker detection in BAL and PLASMA using LUMINEX platform

Bronchoscopies were performed on 20 TB patients at diagnosis and at the end of treatment. During the procedure, bronchoalveolar lavage (BAL) was collected by washing the lung interior with saline. The BAL samples were spun down and the supernatants were used for protein analysis. In addition, blood was collected from these individuals into EDTA tubes and centrifuged in order to collect plasma. Bronchoscopies were also performed on 10 individuals with lung cancer, to serve as a control group. A custom Human $CD8^+$ T-cell Magnetic Bead Panel Kit (HCD8MAG15K17PMX, Merck Millipore, St. Charles, Missouri, USA) was utilised to evaluate the levels of the following six host markers; Granzyme A, Granzyme B, Perforin, soluble Fas Ligand (sFasL), IL5 and Granulocyte macrophage colony stimulating factor (GM-CSF). The assay was carried out according the manufacturer's instructions and the concentration of the marker measured on the MAGPix platform (Bio-Rad Laboratories, California, US). Two quality controls included in the kit were run in duplicate. Levels of all analytes in the quality controls were within the expected ranges. A standard curve ranging from 0.08 to 1.7 pg/ul was used for GM-CSF, 0.93 to 19 pg/ul for Granzyme A, 0.04 to 0.85 pg/ul for Granzyme B, 0.03 to 0.67 pg/ul for IL-5, 0.27 to 6.0 pg/ul for sFasL, and 0.24 to 5.6 pg/ul for Perforin. Bio-Plex Manager software, version 6.1 (California, US), was used to analyse the data.

3.3.6. Statistical analysis

Statistical analysis was carried out using Graph Pad Prism 5 Software (San Diego, CA).Where applicable, differences between groups were calculated using Student t-test, one-way ANOVA and Dunn's multiple comparison post-hoc tests. Significant differences are indicated by $p<0.05$.

3.4. Results

3.4.1. Activated B-cells express a FasL^{+}IL5Ra^{+} phenotype following anti-TB treatment

Seeing that the regulatory "killer" B-cells express CD178 (FASL) and studies have shown that there is an increased expression of IL5RA on these cells, we were interested in the expression of FASL (CD178) and IL5RA (CD125) on B-cells. Additionally, we were interested in the expression of PD1 and CD40 as well, as increased PD1 and CD40 expression is linked to cell exhaustion. To evaluate the expression of the surface markers, the gating strategy shown in Figure 3.1 was followed. When comparing the frequency of CD19^{+} IgM^{+}CD38^{+} cells expressing FASL (CD178) and IL5RA (CD125) between CTRLs and TB cases, there were no significant differences between the two groups (Figure 3.2 a). There was, however, a trend that the expression of FASL (CD178, p=0.06), was higher in CTRLs when compared to TB cases. When comparing the frequency CD19^{+} IgM^{-}CD38^{+} cells expressing FASL/CD178 and IL5RA/CD125, we found that there was a significant difference in the frequency of IL5RA between CTRL and TB groups (p= 0.03; Figure 3.2b). There were no significant differences in frequency of CD19^{+} IgM^{+}CD38^{+} and CD19^{+} IgM^{-}CD38^{+} expressing CD40 and PD1 (Figure 3.2 c and d).

There was, also, however a trend in that the expression of CD40 by IgM^{-} B-cells, was higher in CTRLs (p=0.06). Overall, the expression of FASL/CD178 and IL5RA/CD125, as well as CD40 and PD1 was higher in CTRLs compared to TB participants (Figure 3.2), even though these differences were not statistically significant.

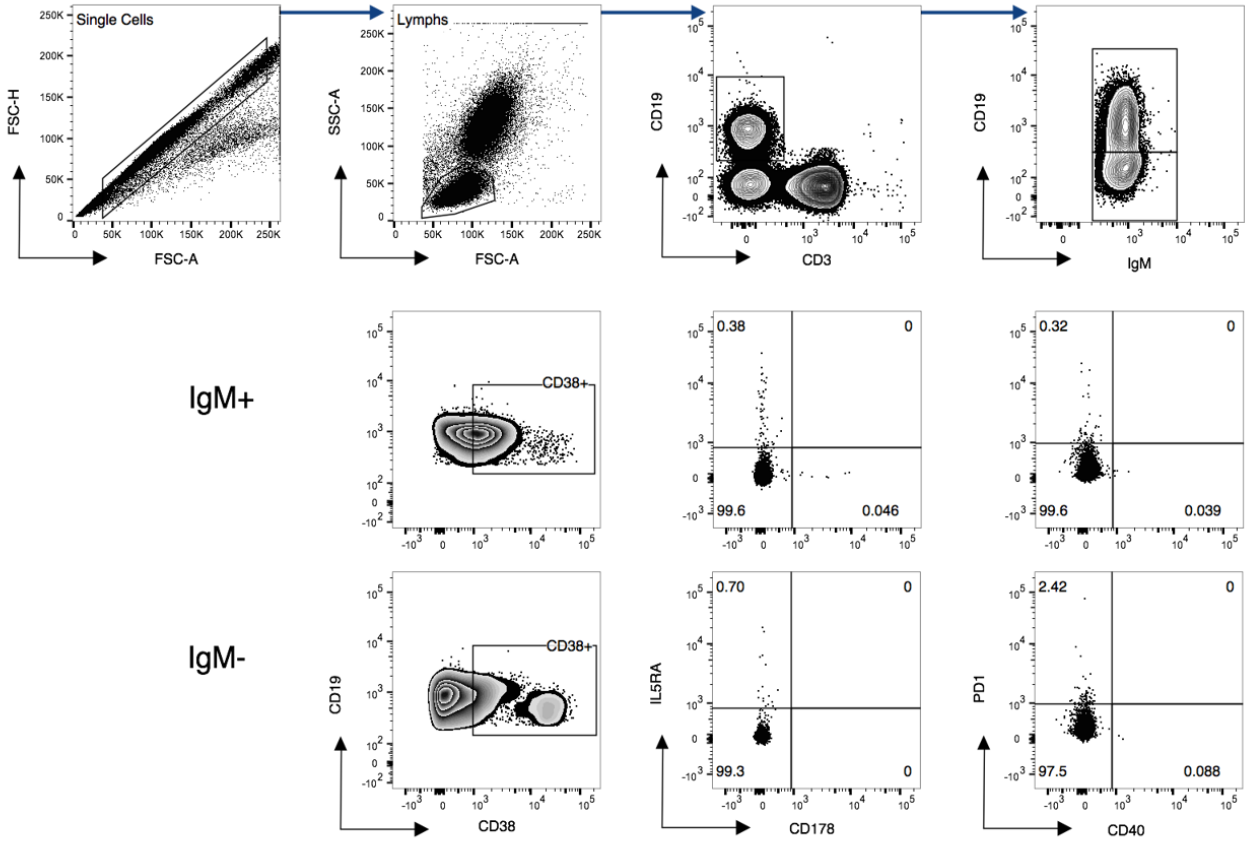


Figure 3.1. The Gating strategy used to identify B-cells of interest
All single cells were selected to exclude doublets, and subsequently lymphocytes were gated on SSC and FSC. $CD3^{-}CD19^{+}$ cells were then gated to select B-cells. Furthermore IgM^{-} and IgM^{+} B-cells were gated and assessed CD178/IL5RA and PD1/CD40 expression.

3.4.2. Effects of anti-TB treatment on the expression of Regulatory and exhaustion markers by B-cells

The frequency of $CD19^{+}IgM^{+}CD38^{+}$ and $CD19^{+}IgM^{-}CD38^{+}$ cells expressing FASL/CD178 and IL5RA/CD125, as well as CD40 and PD1 were evaluated during the course of 6-month TB treatment in order to assess the effects of treatment. The frequency of $CD19^{+}IgM^{+}CD38^{+}$ of cells expressing FASL/CD178 ($CD178^{+}IL5RA^{-}$) decreased during treatment, whereas the expression of IL5RA/CD125 ($CD178^{-}IL5RA^{+}$) increased during treatment (Figure 3.3a). Furthermore, the frequency of cells expressing both FASL/CD178 and IL5RA/CD125 ($CD178^{+}IL5RA^{+}$) was higher at week 2 and month 6 of treatment compared to diagnosis (Figure 3.3a). These changes

were however not statistically significant. The frequency of $CD19^{+}IgM^{-}CD38^{+}$ cells expressing FASL/CD178 decreased by month 6 of treatment, however expression was highest at month 1 of treatment (Figure 3.3b).

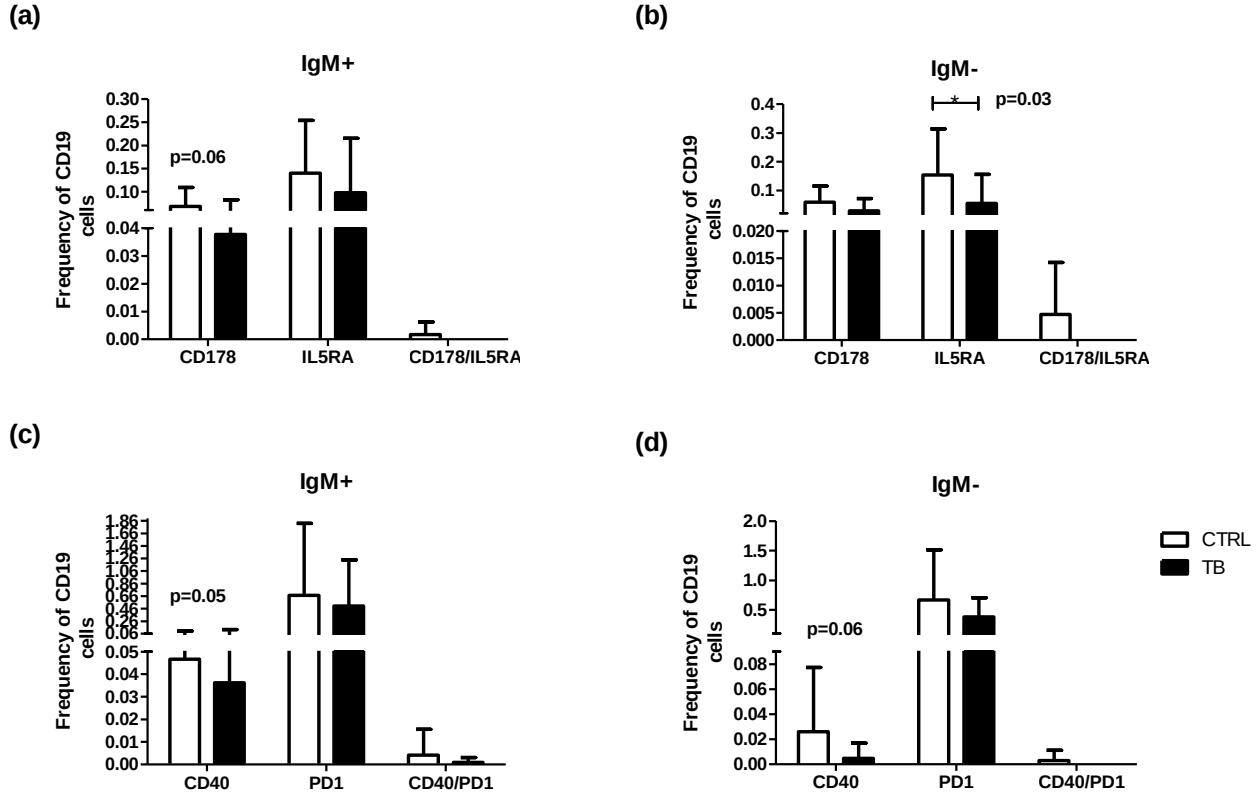


Figure 3.2. Frequency of IgM^{-} and IgM^{+} B-cells expressing FASL (CD178)/IL5Ra (CD125) and CD40/PD1 between TB cases and CTRLs

$CD19^{+}$ B-cells acquired by means of Flow cytometry. Differences in frequency of cells expressing regulatory and exhaustion markers calculated using one-way ANOVA tests and Dunn's multiple comparison post-hoc tests. Significant differences are indicated by an asterisk (*), where $p<0.05$ **(a)** IgM^{+} B-cells expressing FASL/CD178 and IL5RA/CD125 **(b)** IgM^{-} B-cells expressing FASL/CD178 and IL5RA/CD125 **(c)** IgM^{+} B-cells expressing CD40 and PD1 **(d)** IgM^{-} B-cells expressing CD40 and PD1

The expression of IL5RA/CD125 increased by the end of treatment, but was lowest at month 1 of treatment. (Figure 3.3b). Additionally, the frequency of cells expressing FASL/CD178 and IL5RA/CD125 ($CD178^{+}IL5RA^{+}$) was higher at month 1 and month 6 of treatment compared to diagnosis. These changes, however, were not statistically significant either.

The expression of CD40 and PD1 by $CD19^{+}IgM^{+}CD38^{+}$ and $CD19^{+}IgM^{-}CD38^{+}$ cells were evaluated as well (Figure 3.4). The frequency of IgM^{+} and IgM^{-} B-cells expressing CD40 ($CD40^{+}PD1^{-}$) increased during treatment, with the increase being more apparent in the IgM^{+} population, while the frequency of PD1 expressing ($CD40^{-}PD1^{+}$) B-cells decreased (Figure 3.4a and 3.4b). The frequency of IgM^{-} B-cells expressing both CD40 and PD1 ($CD40^{+}PD1^{+}$) was elevated at month 1, however, none of the latter changes were statistically significant.

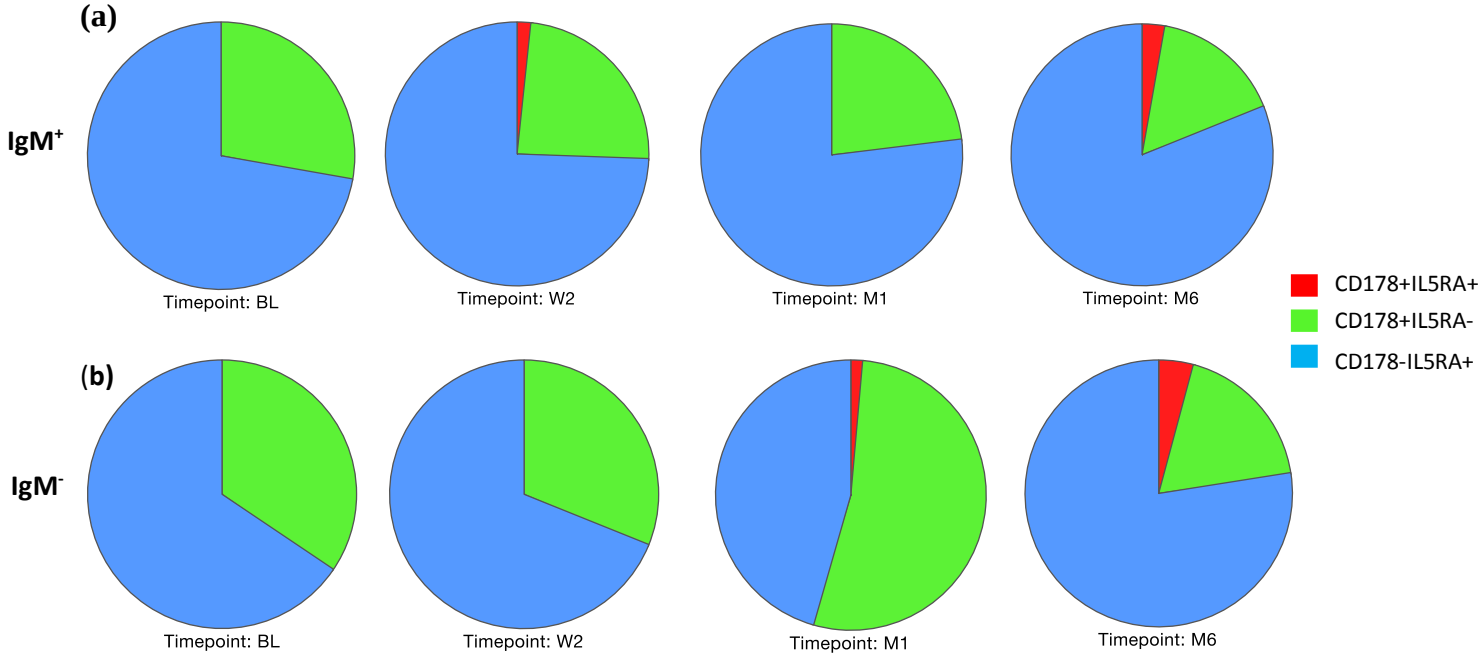


Figure 3.3. Frequencies of Regulatory B-cells expressing CD178 and IL5A during treatment
Pie charts depict frequencies of B-cells expressing either CD178, IL5RA or both simultaneously in TB participants during treatment. Where BL is diagnosis, W2 is week 2, M1 is month1 and M6 is month 6 of treatment. **(a)** Frequencies of IgM positive B-cells and **(b)** Frequencies of IgM negative B-cells

3.4.3. Expression of genes linked to regulatory phenotype during treatment

RT-qPCR was used to evaluate the expression of *FASL* and *IL5RA* transcripts in B-cells from TB participants during treatment (diagnosis, month 2 and month 6). When the expression of genes were compared between the 3 time points, *FASL* expression was found to be significantly upregulated at month 6 compared to diagnosis (p=0.01; Figure 3.5a). Similarly, the expression of *IL5RA* was significantly upregulated at month 2 (p=0.01) and month 6 (p=0.01; Figure 3.5b) compared to diagnosis.

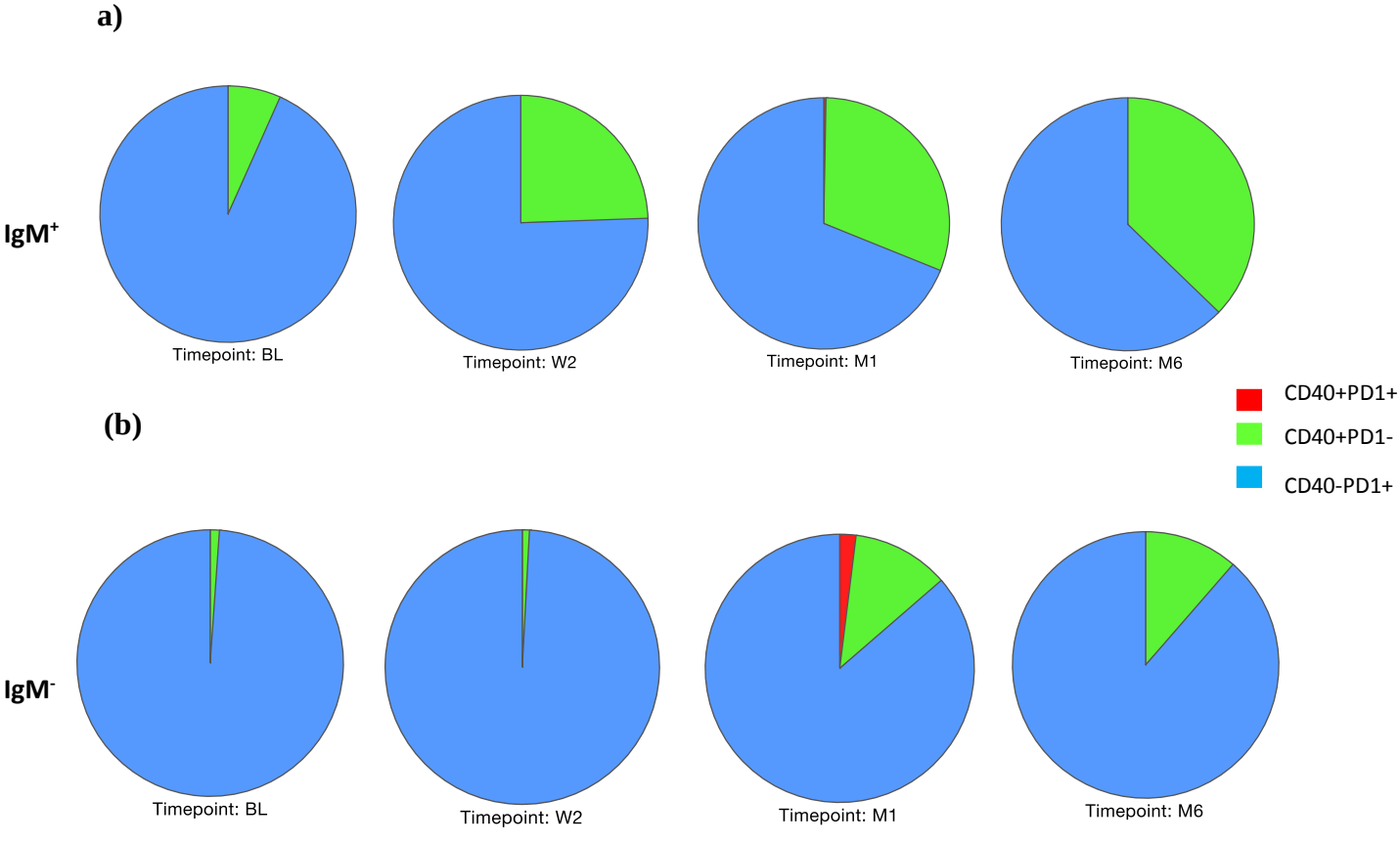


Figure 3.4. Frequencies of Regulatory B-cells expressing CD40 and PD1 during treatment
Pie charts depict frequencies of B-cells expressing either CD40, PD1 or both simultaneously in TB participants during treatment. Where BL is diagnosis, W2 is week 2, M1 is month1 and M6 is month 6 of treatment. **(a)** Frequencies of IgM positive B-cells and **(b)** Frequencies of IgM negative B-cells.

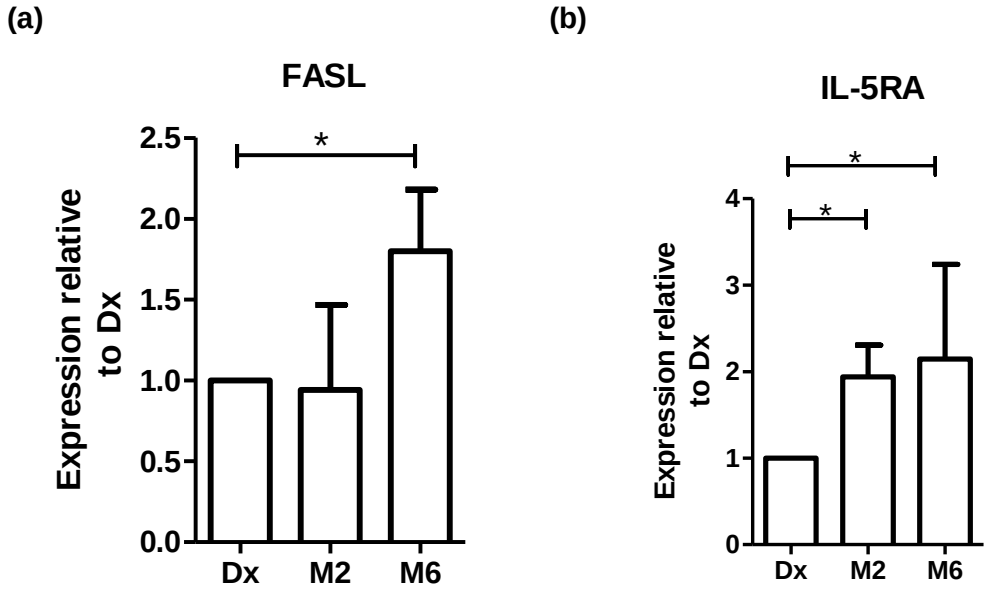


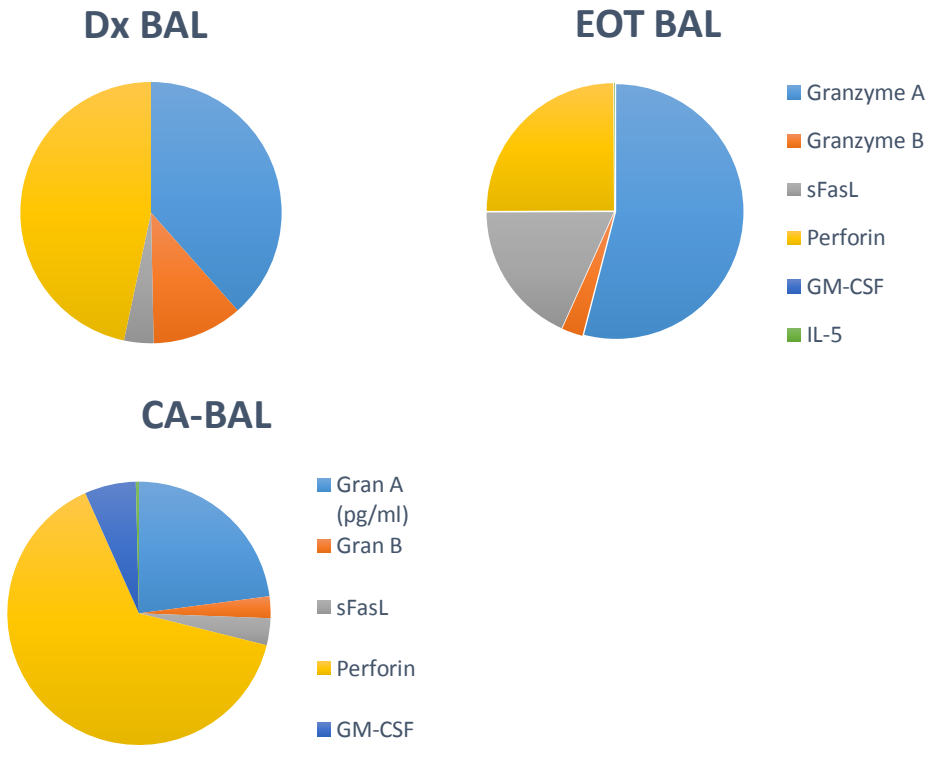
Figure 3.5. Genes differentially expressed during anti-TB treatment
Relative expression of genes as calculated using the delta-delta Ct method. Statistical differences calculated by means of Student t-test, where significant differences are indicated by an asterisk (* = $p \leq 0$, 05). Data represented as mean fold change with bars representing SEM. Positive values indicate increases in gene expression, and negative values indicate decreases in gene expression relative to diagnosis.

3.4.4. Changes in BAL-secreted cytokines following anti-TB treatment but not in the plasma of TB cases

The levels of 6 analytes (Granzyme-A, Granzyme-B, Perforin, sFasL, IL5 and GM-CSF) were measured in the BAL fluid and plasma of TB patients at diagnosis and at the end of treatment (EOT), as well as controls using Luminex technology. When comparing the analyte profiles measured at diagnosis and EOT in the BAL fluid of TB participants, we found that the profiles of the two different time points are different from each other (Figure 3.6a). Furthermore, the analyte profiles of BAL fluid of TB participants at both time points differed from that of CA controls (Figure 3.6a). When comparing the profiles in the plasma of these patients at diagnosis and EOT, no differences were seen (Figure 3.6b). Statistical analysis on the analyte levels in the BAL fluid of the TB patients and CTRLs revealed, that Granzyme-B ($p<0.01$) and Perforin ($p<0.05$) levels are significantly lower at EOT when compared to Dx (Figure 3.7b and d). EOT levels of

Granzyme-B ($p<0.05$), sFasL ($p<0.01$) and Perforin ($p<0.05$) were also significantly different from CTRLs (Figure 3.7b, c and d).

(a)



(b)

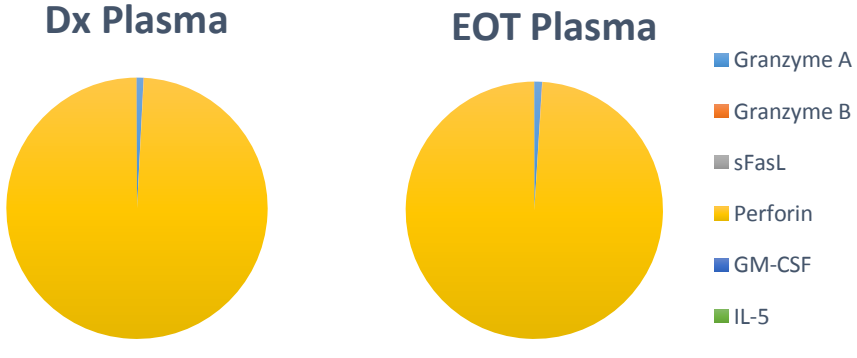


Figure 3.6. Comparison of analyte levels in BAL and Plasma from TB cases at diagnosis and end of treatment

Levels of the analytes in BAL and plasma are represented as percentages within the respective groups. **(a)** Analyte composition in BAL from TB cases at diagnosis (Dx) and end of treatment (EOT), and **(b)** Analyte composition in Plasma from TB cases at Dx and EOT.

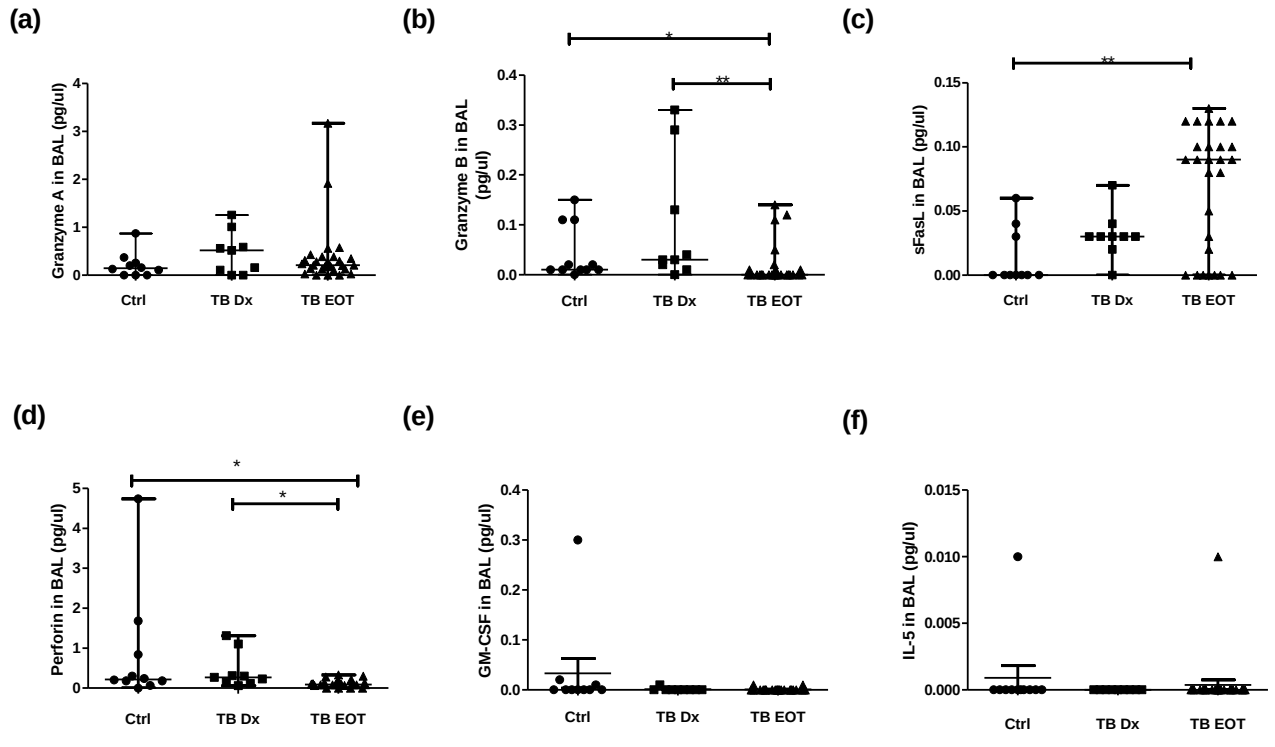


Figure 3.7. Comparison of analyte levels in BAL between Ctrls, TB participants at diagnosis and end of treatment.
Analyte levels (pg/ul) were measured using LUMINEX analysis. ANOVA tests were performed to determine significant differences ($p \leq 0.05$) between groups, which is indicated with an asterisk (*). **(a)** Granzyme A levels **(b)** Granzyme B levels **(c)** sFasL levels **(d)** Perforin levels **(e)** GM-CSF levels and **(f)** IL-5 levels in the respective groups. Bars represent medians and ranges.

3.5. Discussion

The aim of this study was to investigate whether the so-called regulatory "killer" B-cells are present during tuberculosis and the effects of treatment on the frequency of these cells. Literature indicates that these cells are present during autoimmune disease and may play an essential role in the regulation of inflammation [65], [66]. Furthermore, there are characteristics about the regulatory "killer" cells that lead us to think that these cells may play a role during tuberculosis as well, such as the induction of these cells by binding of *M.tb* CpG motifs to TLR9 [47].

The phenotype of B-cells was assessed by means of flow cytometry to identify cells expressing FASL and IL5RA, which are markers associated with and used to identify killer B-cells [64]. Even though there were no significant differences, apart from the difference in the frequency of IgM^- cells expressing IL5RA, the data suggests that there is a higher expression of FASL and IL5RA by cells from CTRLs compared to TB cases. Interestingly, there is an appearance of IgM^- cells expressing both FASL and IL5Ra at month 1 of treatment, which increases by M6. The expression of FASL and IL5RA in IgM^+ cells also increases during treatment, but appears earlier (week 2) during treatment. These results indicate the treatment may be inducing an increase in the expression of FASL by B-cells and that higher expression is associated with reversion back to healthy status, as the data shows that B-cells from healthy controls has a higher expression of FASL compared to TB cases at diagnosis

To assess B-cell activation and exhaustion, we evaluated frequency of B-cells expressing CD40 and PD1. IgM^+ B-cells expressing CD40 increased over time, whereas IgM^- B-cells expressing CD40 only started increasing by month 1 of treatment, with lower frequencies compared to IgM^+ B-cells. This indicates that there is an increase in the activation of B-cells during treatment, which implies that treatment may be inducing activation of B-cells. Cliff and colleagues showed that B-cell associated activity increases as treatment progresses [48].

To assess the effects of anti-TB treatment on the expression of FASL by B-cells at the gene level, we evaluated the changes in transcript levels over time. The results indicated that there is an increase in the levels of both genes by month 6 of treatment. These results correlate to the phenotypic results which indicates that there is an increase in the surface expression of FASL and IL5RA during treatment, even though those changes are not significant.

As a representation of the site of infection, analyte levels in BAL was evaluated by means of the Luminex. sFasL increased by the end of treatment, whereas perforin and granzyme-B levels were

lower at the end of treatment. The increase in sFasL in BAL by the end of treatment also correlates to the phenotypic and gene expression results. FasL expression is mostly attributed to $CD8^+$ T-cells and NK cells in response to viral infections [67], [68]. Although B-cell frequencies in the lung is low, there is increased infiltration of B-cells into the lung during inflammation [69]–[72]. Thus, B-cells may also be contributing to the sFasL levels in the lung.

Collectively, these results show that treatment induces an increase in the expression of FASL by B-cells. These results are in agreement with studies which show that there is an increase in expression of FASL by B-cells during parasitic infection and autoimmune disease [63], [73]. Lundy and colleagues (2001) show that B-cells from mice infected with *Schistosoma mansoni* had a higher expression of FASL compared to healthy mice [63]. Similarly, another study shows an increase in B-cell FASL expression during airway inflammation and asthma, as well as systemic lupus erythromatosis (SLE) [73]–[75]. Increased FASL expression by B-cells may have different effects, depending on the disease or infection. Culture experiments and killing assays carried out by Klinker and colleagues showed that B-cells stimulated with CD40L and IL5 induced FasL-dependent apoptosis of $CD4^+$ T-cells, and that these B-cells target T-cells based on the specificity of the antigen [64]. Thus, increased FASL expression may have a protective role in the case of autoimmune disease, where apoptosis of $CD4^+$ T-cells leads to decreased inflammation. In contrast, apoptosis of Th1 cells may cause a skewed balance between Th1 and Th2 cells, leading to airway inflammation and asthma due to cytokines produced by Th2 cells.

Here, the increase in the expression of FASL during treatment implies that these regulatory "killer" B-cells may play a protective role during TB. One hypothesis may be that these regulatory B-cells are inducing apoptosis of infected T-cells via FASL, as a means to rid the host of the bacteria. An alternative hypothesis may be that these regulatory B-cells are somehow inducing activation of T-cells, which later undergoes apoptosis [76], [77]. Literature states that FasL plays

a role during activation-induced cell death [76], [78]–[80]. When T-cells are activated, there is an increased expression of Fas by these cells and they become sensitized to FasL-induced apoptosis [79]. However, functional studies are required to further elucidate the role of the "killer" B-cells during TB.

Acknowledgements

We thank the participants without who this study would not be possible. We also thank the field staff for participant recruitment and the laboratory personnel of SU-IRG for processing the samples.

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Conflict of Interest

The authors declare no commercial or financial conflict of interest

Chapter 4. B- and T-cell interactions in the context of *M.tb* exposure

4.1. Introduction

Tuberculosis research has come a long way and studies focussing on how the host immune system responds to infection with *Mycobacterium tuberculosis* has indicated that T-cells are at the forefront of the fight against these invading pathogens. Several studies show that $CD4^+$ T-cells correlate with disease progression and that cytokines produced by these cells are essential for the fight against *M.tb* [35], [36], [81]. More specifically, $CD4^+$ T-cells that produce IFNγ, TNFα and IL2 simultaneously, have been associated with protection against *M.tb* [35].In contrast though some reports show that these multifunctional T-cells do not correlate with protection, but with disease (i.e. distinguish between TB cases and latent infection) [36].

Many factors are required for T-cell differentiation and homing to a specific lineage. For instance, antigen presentation cells (APCs) play a big role in T-cell behaviour, as well as the micro-environment. If the micro-environment contains IL12, naïve T-cells will be induced to differentiate into the Th1 lineage and produce proinflammatory cytokines such as IFNγ, TNFα and IL2. Similarly, if the micro-environment contains IL4, naïve T-cells will differentiate into the Th2 lineage and produce anti-inflammatory cytokines such as IL4 [14].

As previously mentioned, APCs play a major role in T-cell activation and differentiation. T-cells require two signals for activation, one of which is provided by the APC. B-cells act as APCs as well, and are able to provide this costimulatory signal to T-cells for activation [12]. However, evidence exists that suggest that this is not the extent of the relationship between B- and T- cells, and that B-cells may further influence T-cell function, such as the frequency of effector and regulatory T-cells. This may be by means of B-cell derived cytokines, or even surface factors [21], [22], [52], [82]. Studies indicate that B-cells play a role in the modulation of $CD4^+$ T-cell

responses. This is mostly shown in the context of autoimmune disease and parasitic infections [82], [83]. Thus, the hypothesis of this study is that B-cells play a role in T-cell modulation during exposure/infection with BCG. The aim and objective of this study is to investigate how B-cells affect T-cell phenotype and function by co-culturing T-cells with autologous B-cells that have been stimulated under various conditions.

4.2. Materials and Methods

4.2.1. Ethics statement

Ethical approval was obtained from the human research ethics committee (HREC) of Stellenbosch University and the City of Cape Town City Health (Ethics number N10/01/013). The study was conducted according to the Helsinki Declaration and International Conference of Harmonisation guidelines. Written informed consent was obtained from all study participants.

4.2.2. Participant recruitment and sample collection

For the purpose of this pilot study, 5 individuals with a QuantiFERON (QFN) positive status were recruited. These participants did not present any clinical symptoms of TB, and QFN status was indicative of exposure to *M.tb*. Additionally, 5 healthy individuals with a negative QFN status were recruited. All participants for this study were HIV negative.

Peripheral blood (40ml) was collected from these individuals from which peripheral blood mononuclear cells (PBMCs) were isolated using the ficol-histopaque method. Subsequently, B-cells were negatively isolated using MACS isolation kit II (Miltenyi Biotec, Germany) according to the manufacturer's instructions. Additionally, T-cells were negatively isolated using the Pan T-cells MACS isolation kit (Miltenyi Biotec) according to the manufacturer's instructions.

4.2.3. B- and T-cell co-culture

Purified B-cells were stimulated with BCG at a concentration of $1.25x10^6$ CFU/ml (with or without other stimulants) in a 96-well round-bottom plate for three hours in an incubator at 37˚C and 5% CO_2 in order to activate the cells. Further culture conditions are shown in Table 4.1. Where applicable, IL5 (Sigma, United States) was added at 50ng/ml and CD40L (Sigma, United States) at 2ug/ml. Subsequently, the cells were centrifuged at 1000rpm for 1 minute and supernatants were removed. Isolated T-cells were then added to the activated B-cells in a ratio of 2:1, and cultured in an incubator at 37˚C and 5% CO_2 for 20hours. RPMI 1640 medium (Sigma, United States) was used for all culture conditions, supplemented with 10% L-glutamine. Following the incubation, the cultured cells were cryopreserved in 90% FCS 10% DMSO and placed in a Mr. Frosty overnight at -80˚C and transferred to liquid nitrogen the following day.

Table 4.1. Culturing conditions for B- and T-cells

1. T-cells only
2. T-cells + BCG
3. B-cells only
4. B-cells + BCG
5. B-cells + T-cells
6. B-cells + BCG + T-cells
7. B-cells + CD40L + IL5
8. B-cells + CD40L + IL5 + T-cells
9. B-cells + BCG + CD40L + IL5 + T-cells

4.2.4. B- and T-cell Flow Cytometry

Cultured cells were retrieved from liquid nitrogen, quickly thawed and washed in FACS buffer containing PBS (Biowhittaker, Belgium) and 2% heat-inactivated Fetal Calf Serum (FCS). The cells were then stained for 30 minutes with the following Beckton Dickenson (BD) antibodies for T-cell-specific analysis; CD3-FITC, CD4-HV500, CD8-APC-Cy7, CD25-PE, IFNg-PE-Cy7, TNF-APC, IL2-PerCP-CY5.5. Additionally, the following antibodies were used for B-cell-specific analysis; CD19-BV510, CD3-PerCP, CD5-APC/Cy7, IgM-FITC, CD24-BV421 (all Identity Bioscience, South Africa), CD38-PE/Cy7, CD125 (IL5RA)-PE and CD178 (FASL)-APC (all Biocom Biotech, South Africa). Subsequently, the samples were acquired on a BD FACS Canto II and the data analysed using FlowJo v10 software. To determine the appropriate gating cut-off and to increase the accuracy of distinguishing different populations, Fluorescence-minus-one (FMO) control samples were utilised (as described by Perfetto *et al*.(2004)) [84]. The background cytokine expression was subtracted from the T-cell stimulation conditions. The different gating strategies for B-cells and T-cell cytokine production can be found in Addendum B.

4.2.5. Statistical Analysis

Statistical differences between groups were calculated using the Mann Whitney U-test, and differences within groups were calculated using the Kruskal-Wallis with Dunns post-hoc tests. Spearman correlation tests were used for relationship analyses. Statistical significance is indicated by an asterisk (*), where $p<0.05$. Prism 6 Software (San Diego, CA) was used for all statistical analysis.

4.3. Results

4.3.1. Regulatory and Memory B-cell frequencies

Evidence exist which indicate that regulatory B-cells (Bregs) suppress effector T (TE) cells during parasite infections and autoimmune disease [83]. Thus, to evaluate the changes in frequencies of Bregs in the context of *M.tb* exposure, we assessed the frequency of $CD19^{+}CD24^{hi}CD38^{hi}$ B-cells between QFN positive and negative groups, and in response to various stimuli. B-cells from QFN negative individuals had a higher frequency of Bregs compared to QFN positive individuals (Figure 4.1a). Similarly, when B-cells were stimulated with either BCG on its own or in combination with CD40L and IL5, there was a higher frequency of Bregs in QFN negative group compared to QFN positive (Figure 4.1a). These differences, however, were not significant.

To evaluate changes in the frequency of memory B-cells (Bmemory), we assessed $CD19^{+}CD24^{hi}CD38^{-}$ B-cells. Overall, Bmemory frequencies were higher in the QFN positive group compared to QFN negative (Figure 4.1b). However, there were no significant differences in Bmemory frequencies between stimulation conditions.

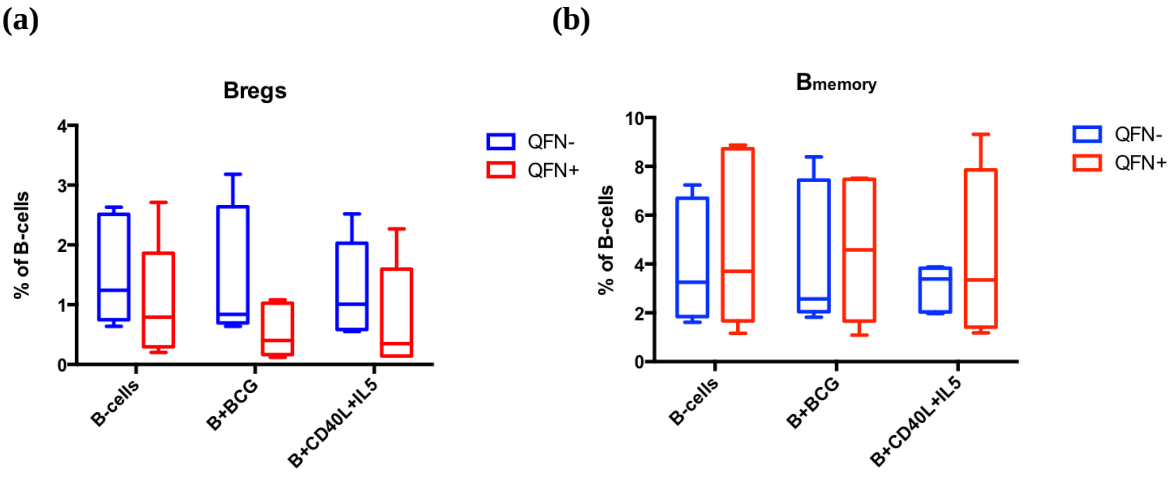


Figure 4.1. Changes in Frequency of Bregs and Bmemory cells.
These cells are expressed as a percentage of $CD19^+$ B-cells, where Bregs are $CD19^+CD24^{hi}CD38^{hi}$ and Bmemory are $CD19^+CD24^{hi}CD38^-$. B-cells were either stimulated with BCG on its own or with CD40L and IL5. Statistical differences between groups were calculate using Mann Whitney U-test, and differences with in groups were calculated using Kruskal-Wallis with Dunns post-hoc tests. **(a)** Frequency of Bregs between QFN positive and negative groups following stimulation and **(b)** Frequency of Bmemory between QFN positive and negative groups after various stimulations.

4.3.2. FasL-expressing B-cells

Klinker and colleagues have shown that culturing naïve B-cells with CD40L and IL5 results in the expression of surface FasL (CD178) and IL5RA by B-cells, which they termed "Killer" B-cells [64]. In order to assess this expression in the context of *M.tb* exposure, we stimulated B-cells from QFN negative and positive individuals with BCG on its own, and in combination with CD40L and IL5. To evaluate the frequency of these "Killer" B-cells, we assessed $CD19^+CD5^+IgM^+CD38^+$ B-cells. Within the QFN positive group, there was a significant increase in the frequency of "Killer" B-cells ($CD178^+IL5RA^+$) following stimulation (p=0.039, Figure 4.2a). There were no significant differences in the frequency of B-cells expressing CD178 only ($CD178^+IL5RA^-$, Figure 4.2b). However, culturing B-cells with CD40L and IL5 resulted in an increase in the frequency of $CD178^+$ B-cells compared to B-cells only within the QFN negative group. Similarly, stimulation of B-cells with CD40L and IL5 resulted in a significant increase in

the frequency of $CD178^{-}IL5RA^{+}$ B-cells (p=0.023, Figure 4.2c). Furthermore, even though not significant, CD40L and IL5 stimulation induced a higher frequency of $CD178^{-}IL5RA^{+}$ B-cells in both QFN negative (ns, p>0.05) and positive groups (ns, p>0.05), compared to B-cells only.

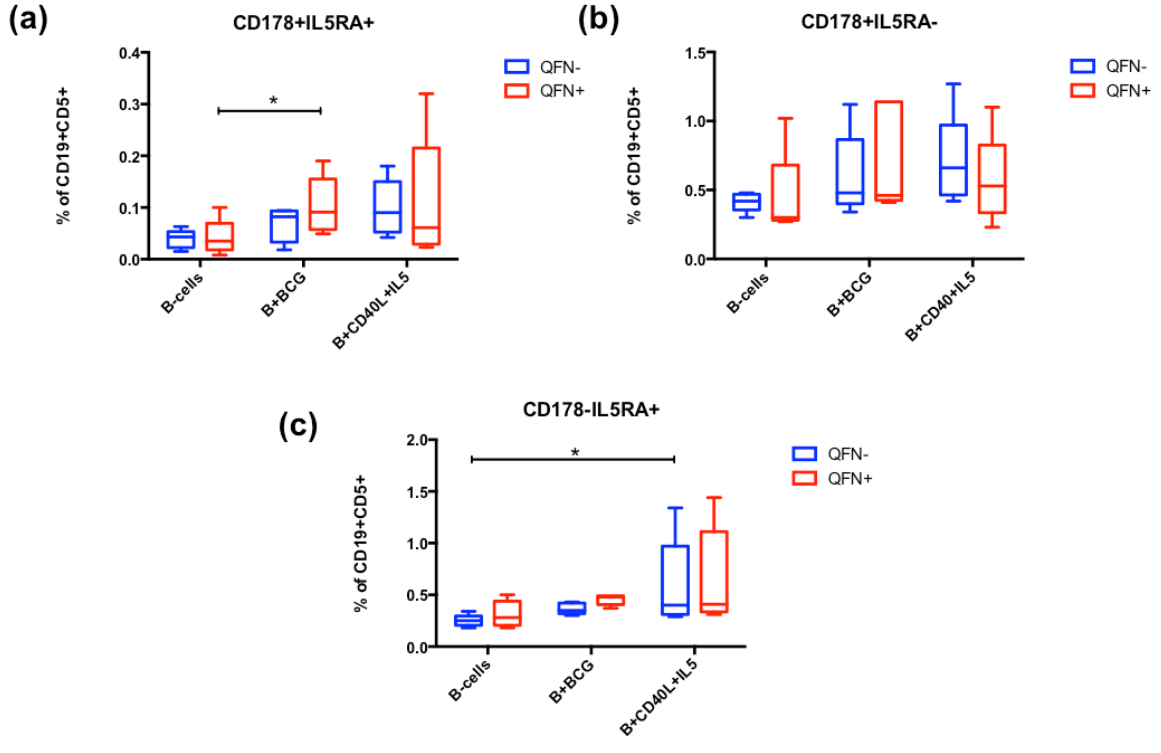


Figure 4.2. Changes in frequency of FasL-expressing B-cells
These cells are expressed as a percentage of $CD19^{+}CD5^{+}$ B-cells. B-cells were either stimulated with BCG on its own or CD40L and IL5. Statistical differences between groups were calculate using Mann Whitney U-test, and differences with in groups were calculated using Kruskal-Wallis with Dunns post-hoc tests. Significant difference is indicated by an asterisk (*) **(a)** Frequency of $CD178^{+}IL5RA^{+}$ B-cells between QFN positive and negative groups after various stimulations **(b)** Frequency of $CD178^{+}IL5RA^{-}$ B-cells **(c)** Frequency of $CD178^{-}IL5RA^{+}$ B-cells.

4.3.3. Effector and Regulatory T-cells

T-cells are essential in the immune response against *M.tb* infection, and there is evidence that B-cells may modulate T-cell phenotypes. Thus, we assessed the phenotype of T-cells following culture with B-cells that have been stimulated under various conditions. More specifically, we evaluated the frequencies of $CD3^{+}CD4^{+}CD25^{+}$ effector T-cells (TEs) and $CD3^{+}CD4^{+}CD25^{hi}$ regulatory T-cells (Tregs).

After T-cells were cultured with B-cells stimulated with BCG, there was a decrease in TEs in both QFN negative and positive groups, compared to T-cells on its own (Figure 4.3a). This was, however not a significant difference. Similarly, there was a significant decrease in the frequency of TEs in the QFN negative group when T-cells were cultured with B-cells stimulated with BCG, CD40L and IL5 ($p=0.01$, Figure 4.3a).

After T-cells were cultured with B-cells that was stimulated with BCG, there was a decrease in Tregs in both QFN negative and positive groups (Figure 4.3b) compared to T-cells cultured on its own. Similarly, when T-cells were cultured with B-cells that were stimulated with CD40L and IL5, there was a decline in the frequency of Tregs in both QFN negative and positive groups. Treg frequency in the QFN negative group was also lower after being cultured with B-cells stimulated with BCG, CD40L and IL5 compared to T-cells cultured on its own ($p=0.03$, Figure 4.3b).

(a) **(b)**

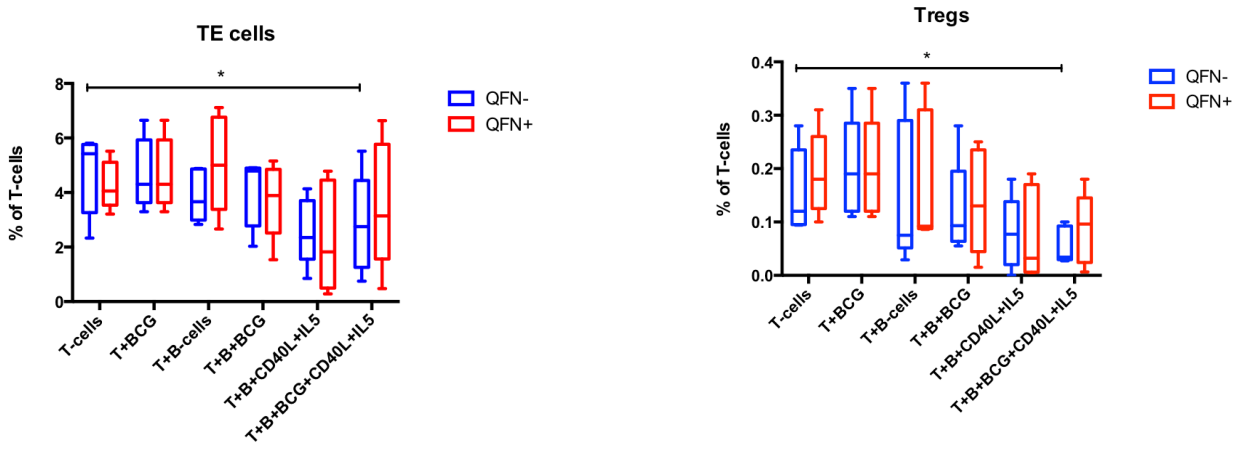


Figure 4.3. Changes in the frequency of TE and Treg cells
These cells are expressed as a percentage of $CD3^+$ T-cells. T-cells were cultured on its own, or with B-cells that were either stimulated with BCG on its own or CD40L and IL5. Statistical differences between groups were calculate using Mann Whitney U-test, and differences with in groups were calculated using Kruskal-Wallis with Dunns post-hoc tests. Significant difference is indicated by an asterisk (*) **(a)** Frequency of $CD3^+CD4^+CD25^+$ T-cells between QFN positive and negative groups after various stimulations **(b)** Frequency of $CD3^+CD4^+CD25^{hi}$ T-cells

4.3.4. Relationship between Bregs and Tregs

Studies show that when Bregs are induced, these cells suppress TEs and induce Tregs. Thus, to evaluate whether this relationship applies in the context of *M.tb* exposure, we assessed the correlation between Bregs and TE, as well as Bregs and Tregs.

There was a positive correlation between Bregs and TEs from QFN positive individuals when B-cells were stimulated with CD40L and IL5 (r=0.97, p=0.03, Figure 4.4e). Furthermore, there was a positive correlation between Bregs and Tregs from QFN positive individuals after B-cells were stimulated with CD40L and IL5 (r=0.97, p=0.03, Figure 4.5e).

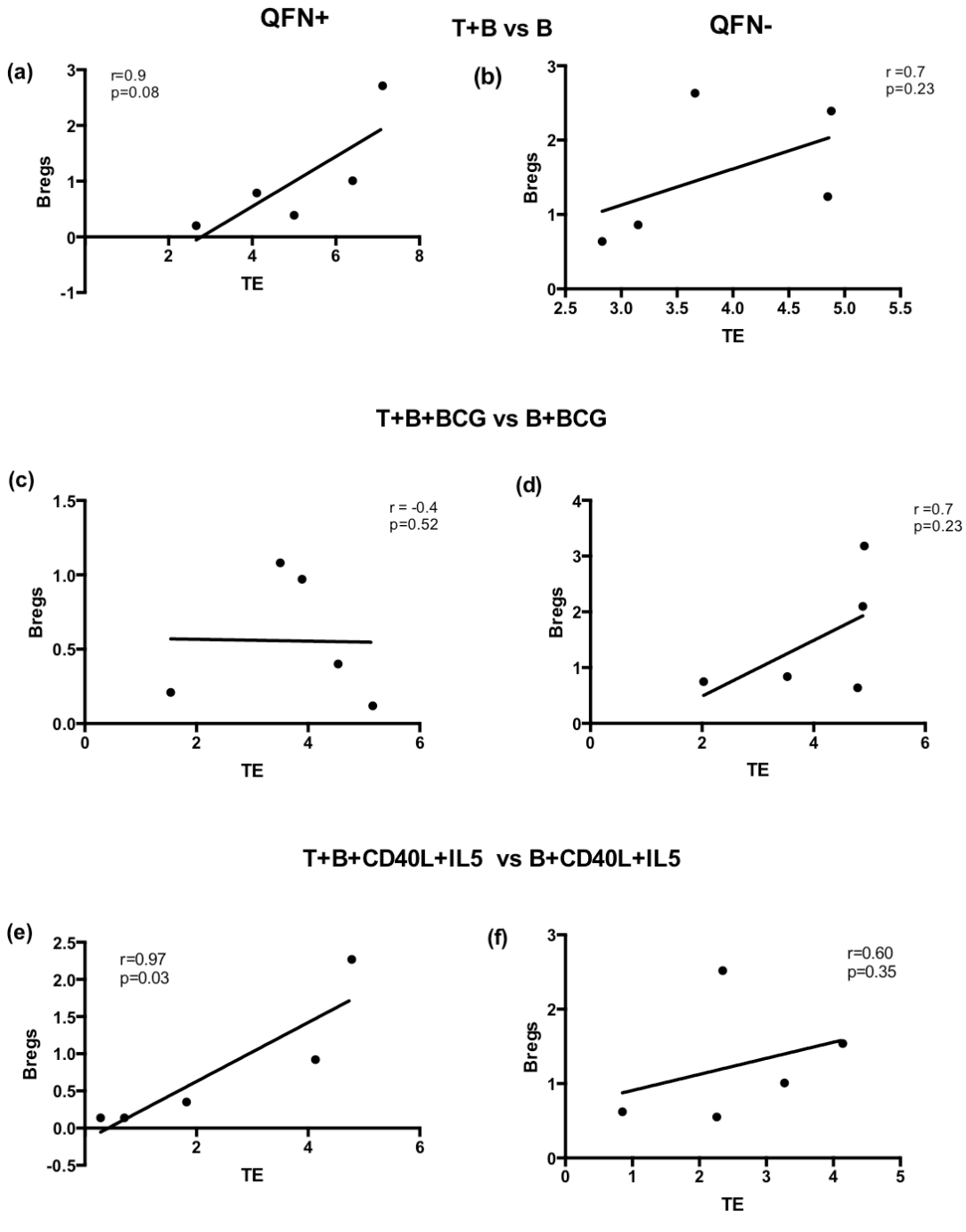


Figure 4.4. Correlation between frequency of Breg and TE cells

Spearman correlation analysis of Bregs and TEs in both QFN positive and negative groups. **(a)** Correlation between Bregs and TE when QFN positive B-cells cultured with B-cells **(b)** Correlation between Bregs and TE when QFN negative B-cells cultured with B-cells **(c)** Correlation between Bregs and TE when T-cells were cultured with BCG-infected B-cells from QFN positive individuals. **(d)** Correlation between Bregs and TE when T-cells were cultured with BCG-infected B-cells from QFN negative individuals **(e)** Correlation between QFN positive Bregs and TE when T-cells cultured with B-cells stimulated with CD40L and IL5 **(f)** Correlation between QFN negative Bregs and TE when T-cells cultured with B-cells stimulated with CD40L and IL5.

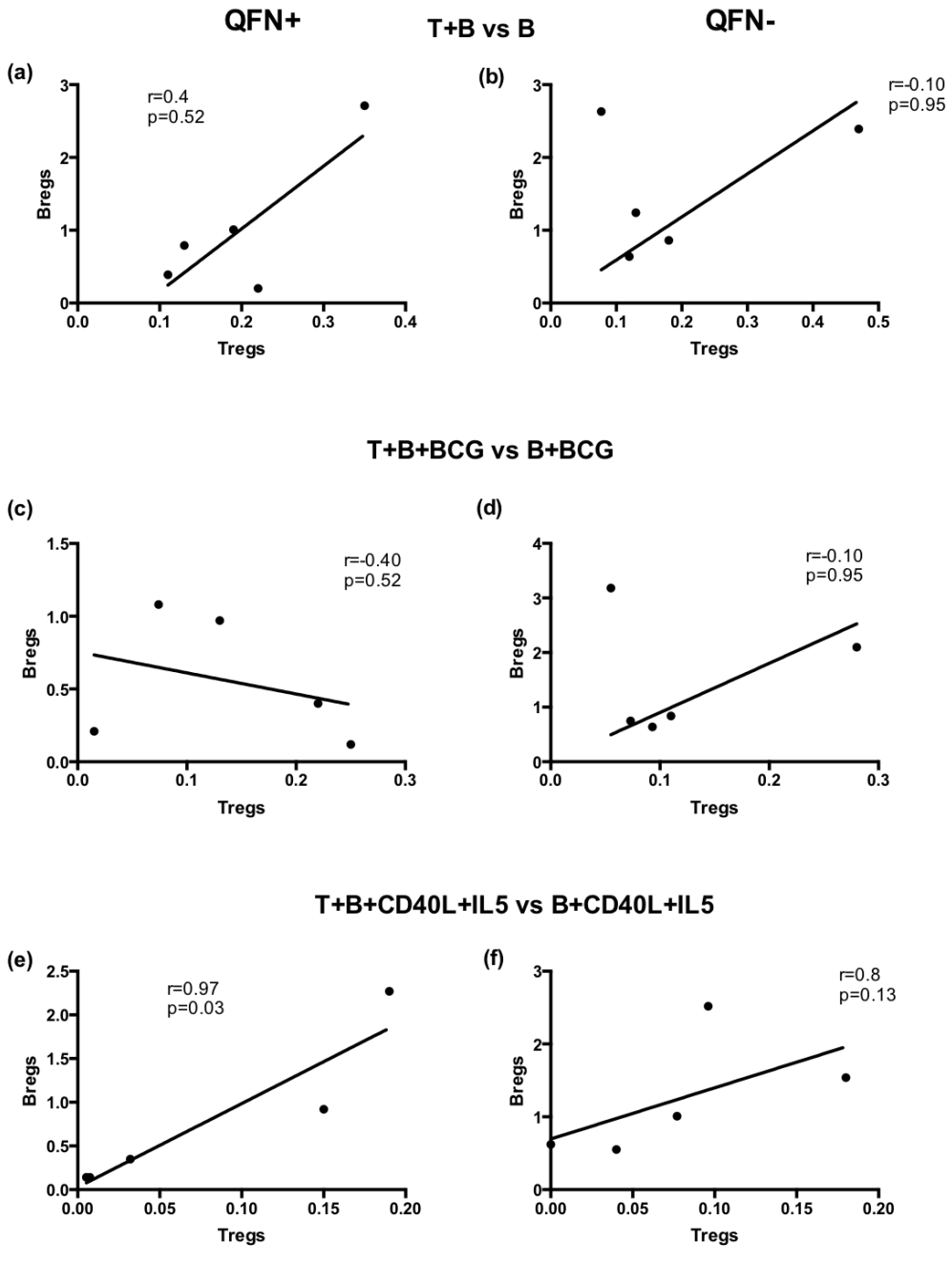


Figure 4.5. Correlation between Frequency of Breg and Treg cells

Spearman correlation analysis of Bregs and Tregs in both QFN positive and negative groups. **(a)** Correlation between Bregs and Tregs when QFN positive B-cells cultured with B-cells **(b)** Correlation between Bregs and Tregs when QFN negative B-cells cultured with B-cells **(c)** Correlation between Bregs and Tregs when T-cells were cultured with BCG-infected B-cells from QFN positive individuals. **(d)** Correlation between Bregs and Tregs when T-cells were cultured with BCG-infected B-cells from QFN negative individuals **(e)** Correlation between QFN positive Bregs and Tregs when T-cells cultured with B-cells stimulated with CD40L and IL5 **(f)** Correlation between QFN negative Bregs and Tregs when T-cells cultured with B-cells stimulated with CD40L and IL5.

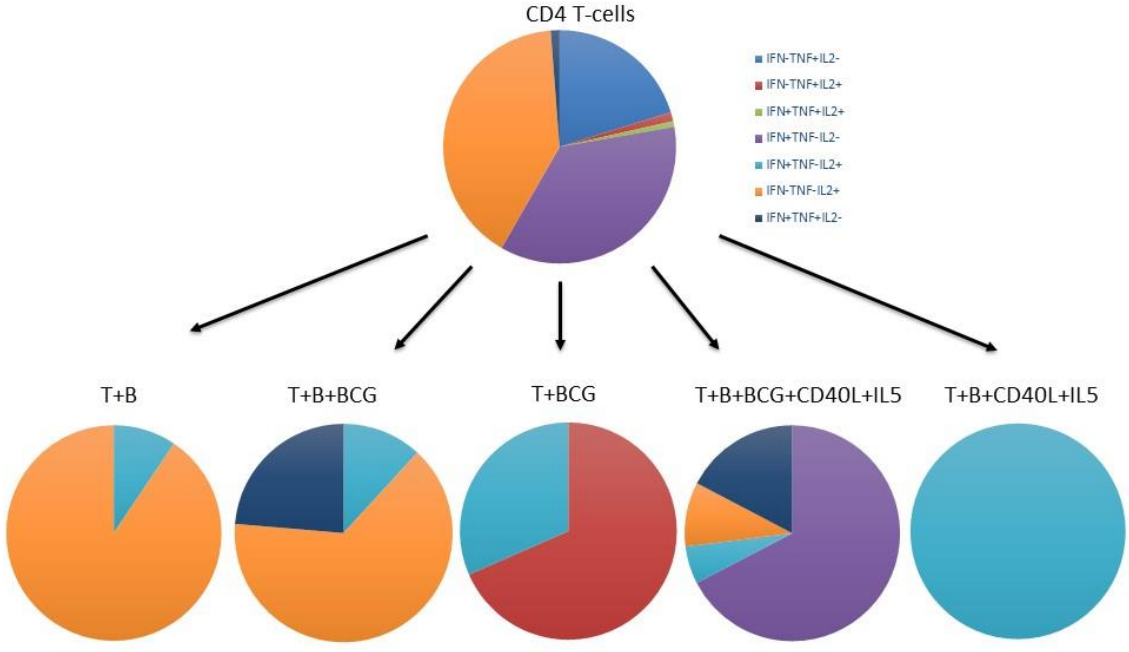
4.3.5. T-cell cytokine production

B-cells produce cytokine which in turn may affect T-cell cytokine production [82]. Thus, in order to investigate the effect of B-cell phenotype on T-cell cytokine production, we evaluated $CD4^+$ and $CD8^+$ T-cell cytokine production following culture with pre-stimulated B-cells.

B-cells from QFN positive individuals that have not been pre-stimulated induced an increase in IL2-producing $CD4^+$ T-cells, as well T-cells that produce both IFNγ and IL2 (ns, $p>0.05$, Figure 4.6a). B-cells that have been pre-stimulated with BCG induced a similar cytokine milieu to unstimulated B-cells, with the addition of $CD4^+$ T-cells that produce IFNγ and IL2 (ns, $p>0.05$). Reports suggest that B-cells stimulated with CD40L and IL5 induced FasL-expressing B-cells [64]. Thus, in order to see the effect of these FasL-expressing B-cells on T-cell cytokine production, we cultured T-cells with B-cells pre-stimulated with CD40L and IL5. When BCG-infected B-cells were stimulated with CD40L and IL5 (i.e. induce FasL expression), there was an increase in $CD4^+$ T-cells that produce IFNγ (ns, $p>0.05$).

Unstimulated B-cells from QFN negative individuals induced an increase in $CD4^+$ T-cells that produce TNFα, IFNγ and TNFα, IFNγ and IL2, and TNFα and IL2 (ns, $p>0.05$, Figure 4.6b). Following BCG infection, the B-cells induced $CD4^+$ T-cells that produce IFNγ and IL2, and IL2 only (ns, $p>0.05$). When BCG-infected B-cells were stimulated to induce FasL, this cytokine milieu changes, where $CD4^+$ T-cells are induced to produce IL2 only, IFNγ and IL2, and IFNγ and TNFα (ns, $p>0.05$).

(a)QFN +



(b) QFN-

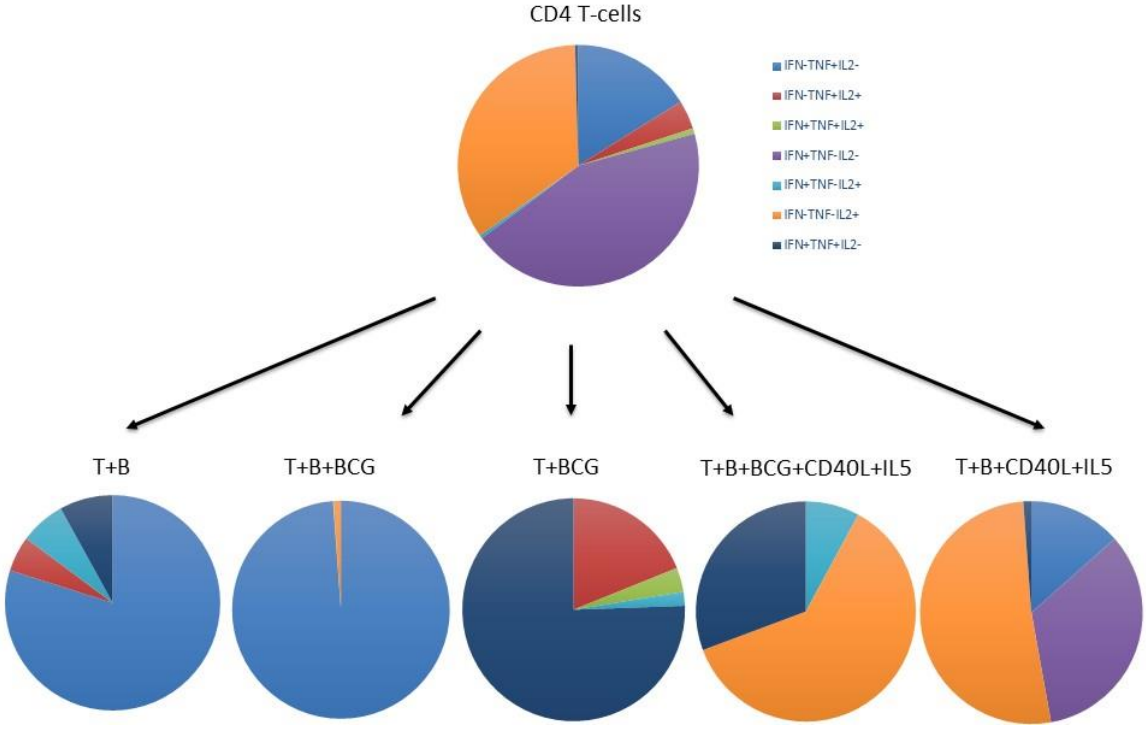
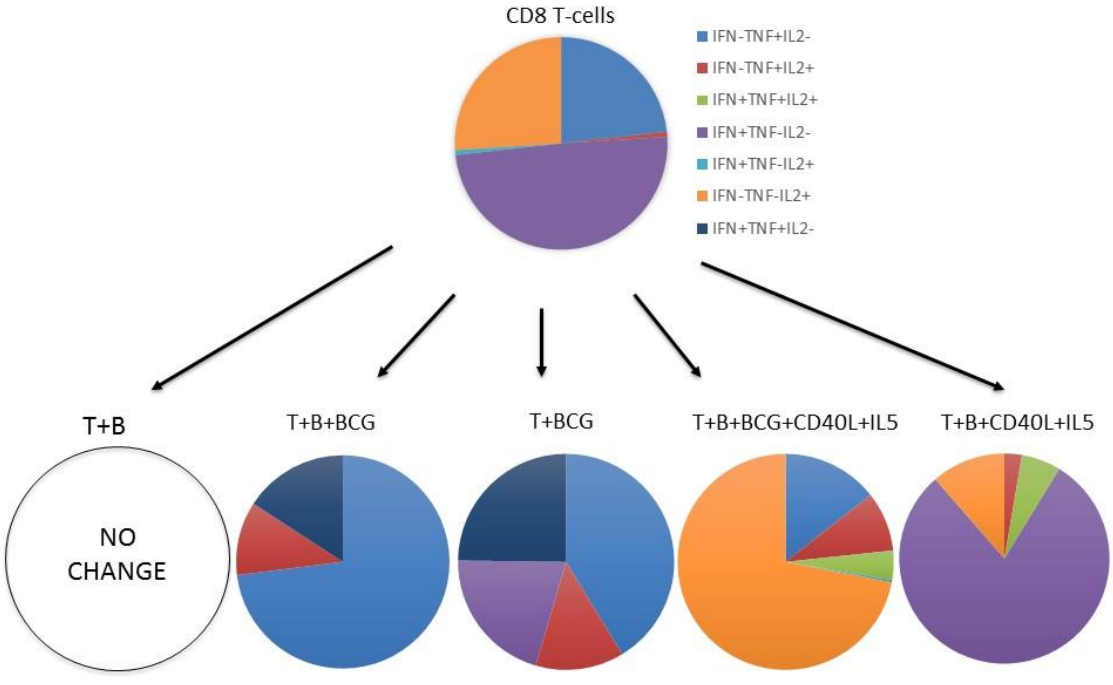


Figure 4.6. Cytokine production by $CD4^+$ T-cells
Change in cytokine production by $CD4^+$ T-cells following various stimulation conditions with B-cells compared to T-cells by itself. T cells only represent background cytokine production, which were subtracted following the different stimulations. The key indicates which cytokine combinations the colours represent. **(a)** Cytokine production in QFN positive group **(b)** Cytokine production in QFN negative group.

Furthermore, $CD8^+$ T-cell cytokine production was evaluated in response to the various culturing conditions. Unstimulated B-cells from QFN positive individuals did not induce any change in the $CD8^+$ T-cell cytokine milieu (ns, $p>0.05$, Figure 4.7a). However, when these B-cells were stimulated with BCG, it induced $CD8^+$ T-cells that produce TNFα, TNFα and IL2, and IFNγ and TNFα (ns, $p>0.05$). Furthermore, following the pre-stimulation of BCG-infected B-cells with CD40L and IL5, there was an increase in $CD8^+$ T-cells producing IL2 only, TNFα only, TNFα and IL2, as well as multi-functional $CD8^+$ T-cells (ns, $p>0.05$).

Unstimulated B-cells from the QFN negative group induced an increase in $CD8^+$ T-cells that produce IFNγ and TNFα, IL2 only, and IFNγ and IL2 (ns, $p>0.05$, Figure 4.7b). When B-cells were stimulated with BCG, it induced an increase in $CD8^+$ T-cells that produce TNFα only, and TNFα and IL2 (ns, $p>0.05$). Furthermore, when BCG-infected B-cells are stimulated to express FasL, it induces $CD8^+$ T-cells to produce IL2 only, IFNγ only, TNFα only, and IFNγ and TNFα (ns, $p>0.05$).

(a) QFN +



(b)QFN-

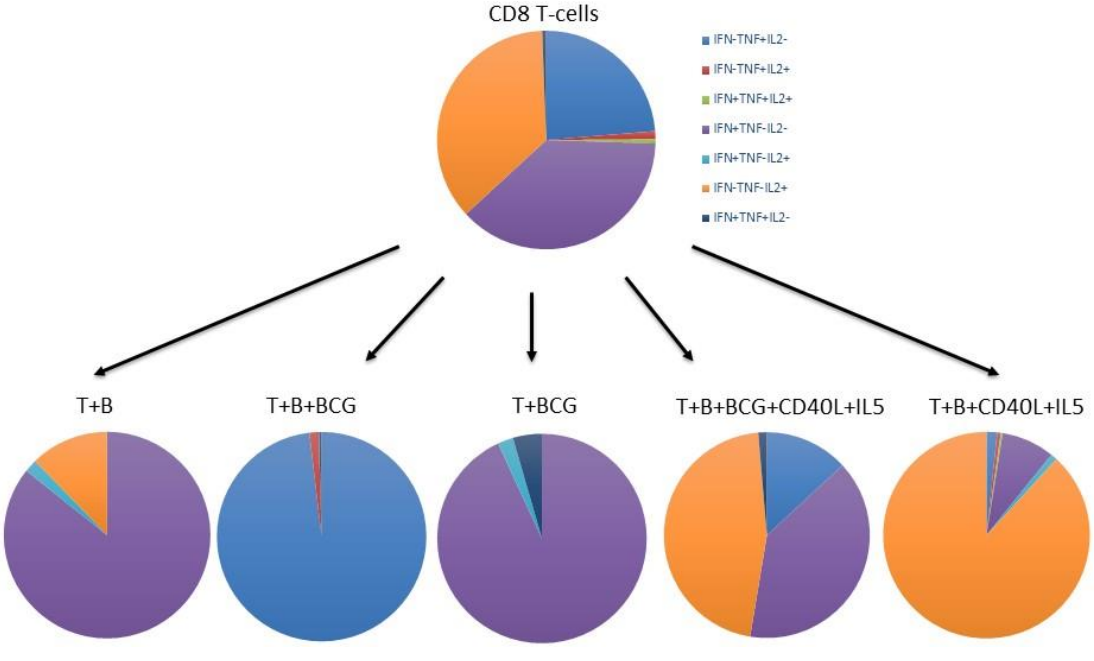


Figure 4.7. Cytokine production by CD8$^+$ T-cells
Change in cytokine production by CD8$^+$ T-cells following various stimulation conditions with B-cells compared to T-cells by itself. T cells only represent background cytokine production, which has already been subtracted following the different stimulations. The key indicates which cytokine combinations the colours represent. **(a)** Cytokine production in QFN positive group **(b)** Cytokine production in QFN negative group.

4.4. Discussion

The aim of this study was to assess how B-cells influence T-cell phenotype and cytokine production in the context of *M.tb* exposure. For the purpose of this study, QFN positive individuals who represent healthy exposed (i.e. latent TB infection) and QFN negative who represent healthy uninfected individuals were used. All these participants were HIV negative. The difference between QFN positive individuals and TB cases is that the immune system of QFN positive individuals were able to contain *M.tb* infection and did not develop active TB disease. Thus, understanding the relationship between B- and T-cells in QFN positive individuals is essential, in order to understand how these cells contribute to overcoming the infection.

Evidence exist that suggest that B-cells may influence T-cell effector functions by means of cytokine production and various phenotypes such as regulatory and FasL-expressing B-cells [21], [22], [52], [82]. Thus, the objectives here were to assess B- and T-cell phenotypes, as well as T-cell cytokine production in response to various stimuli in the context of *M.tb* exposure.

To assess B-cell phenotypes in the context of *M.tb* exposure, and how it compares to healthy individuals, the frequency of regulatory and memory B-cells were evaluated. There were no significant changes in the frequency of regulatory B-cells (Bregs) when stimulated with BCG or CD40L and IL5. Although, Bregs frequencies were higher in QFN negative individuals compared to QFN positive individuals (not significant). These $CD19^{+}CD24^{hi}CD38^{hi}$ Bregs have not yet been investigated during *M.tb* infection or disease. Wang and colleagues (2015) show that these cells are functional during gastric cancer [25]. However, they show that there is no significant difference in the frequency of Bregs between healthy individuals and those with cancer [25]. Furthermore, Joosten and colleagues (2016) showed that the frequency of Bregs are slightly higher

in healthy individuals compared to individuals with latent TB infection, even though this was not statistically significant [85]. This is similar to the results in the current study.

Additionally, the frequency of "killer" B-cells was investigated. The results show that there was an increase in the frequency $FASL^{+}IL5RA^{+}$ "killer" B-cells when stimulated with either BCG or CD40L and IL5 from both QFN positive and negative groups, even though it lacks statistical significance. These FasL-expressing B-cells have not yet been investigated in the context of *M.tb* infection or disease, but rather during autoimmunity and parasite infections. These studies show that there is an increase in the frequency of these FasL-expressing B-cells, which in turn have a beneficial impact as it has a regulatory effect on proinflammatory responses [43], [44], [58], [60].

In part, the results from the current study may indicate a beneficial role for FasL-expressing B-cells due to the increased frequency following BCG stimulation. As discussed in chapter 3, we hypothesise that FasL-expressing B-cells may be inducing FasL-mediated apoptosis of $CD4^{+}$ T-cells or inducing activation of T-cells which later leads to apoptosis due to increased Fas expression by these activated T-cells. Furthermore, results from chapter 3 showed that the control group (LTBI) had a higher expression of FasL compared to TB cases, and anti-TB treatment induced increased expression of FasL in TB cases.

In order to investigate the effect B-cells have on T-cells function, the frequency of effector (TE) and regulatory (Treg) T-cells was evaluated following culturing with B-cells that have been pre-stimulated in various ways. B-cells induced an increase in the frequency of TEs in QFN positive individuals, even though this is not a significant change. This may imply that B-cells contribute to T-cell effector functions by inducing an increase in TEs. B-cells may be producing cytokines that enhances T-cell responses, as proposed by Lund and colleagues (2010) [83]. Here, the fact

that B-cells (unstimulated) from QFN positive individuals having the ability to induce an increase the frequency of TEs is interesting as these individuals have been exposed to *M.tb* but did not go on to develop TB disease. Could the B-cells contribution have enhanced protection against *M.tb* infection? Further experiments and over a longer follow up period are required to elucidate this phenomenon/hypothesis/question.

In contrast, culturing BCG-infected B-cells and B-cells that have been induced to express FasL, with T-cells resulted in a decline in the frequency of TEs (not significant). Stimulating B-cells with CD40L and IL5 induces FasL-expressing B-cells, which is a subset of Bregs. Studies show that these FasL-expressing cells are able to produce IL10 as well, as a means to regulate immune responses. Literature indicates that Bregs suppress TEs by means of producing cytokine such as IL10 or FasL-mediated apoptosis, which supports the results shown here [25], [64].

As previously mentioned, Bregs induces Tregs during autoimmune disease, and suppresses TEs [25], [83]. Here, the correlation analysis indicate that there is a positive relationship between Bregs and Tregs. Similarly, there is a positive correlation between Bregs and TEs as well. These results are only supported in part by literature[25], [83], where one would expect a positive relationship between Bregs and Tregs. In contrast to the results here, one would expect a negative correlation between Bregs and TEs, as literature suggests that TEs should decrease with an increase in Bregs.

The increase in frequency of Tregs have different effects, depending on the stimulation condition. For instance, during autoimmune diseases, it suppresses/dampens the inflammatory responses by effector T-cells but during TB disease it may have a detrimental effect [26], [27], [83], [86], [87]. Murine studies have shown that depletion of Tregs in mice which have been infected with *M.tb* displayed better clearance of bacteria, and that enhanced Treg frequencies led to increased bacterial load [86], [87]. Further studies indicated that Tregs from individuals with active TB

disease dampen T-cell responses to *M.tb* antigen [26], [27]. The latter effects are mostly attributed to $CD4^+CD25^{hi}FoxP3^+$ Tregs, whereas the current study focussed on $CD4^+CD25^{hi}$ Tregs.

Finally, in order to assess the effect of B-cells on T-cell cytokine production, autologous B- and T-cells were co-cultured and $CD4^+$ and $CD8^+$ responses were measured. Despite the statistical insignificance of the results, it implies that B-cells, whether pre-stimulated or not, are able to induce cytokine production by both $CD4^+$ and $CD8^+$ T-cells. These results are in agreement with Soares *et al* (2008) who showed that stimulating blood with BCG induces complex cytokine profiles in T-cells (i.e. combinations of IFNγ, IL2 and TNFα) [88]. Here, B-cells, depending on the antigen or microenvironment, may be secreting cytokines that influence T-cell cytokine production [83], or it may be through contact with B-cells that influenced cytokine production by T-cells. Further experiments are required to elucidate this phenomenon.

Cytokine production by $CD4^+$ T-cells in the context of TB disease has received much more attention than $CD8^+$ T-cells. Our results indicate that B-cells induce cytokine production by $CD4^+$ T-cell as well as $CD8^+$ T-cells in both *M.tb* exposed and unexposed individuals. Researchers have been sceptical about $CD8^+$ T-cells role during TB disease because of the manner in which antigens are presented to these cells. $CD8^+$ T-cells recognise antigen in the context of MHC I, which is loaded in the cytosol [89]. Thus, because *M.tb* is phagocytosed/endocytosed, one would expect that these peptides may only be presented in the context of MHC II and thus $CD4^+$ T-cell responses have been the main focus. However, there is evidence that $CD8^+$ T-cells play a role during active TB [90], but further studies are required to confirm this in human. Furthermore, there is evidence which suggests that $CD8^+$ T-cells are required for the control of *M.tb* infection in latently infected mice [91]. A possible reason for the $CD8^+$ T-cell response is the translocation of the phagosome

to the cytosol, which then leads to peptides being loaded onto MHC I [92], and cross-presentation of peptides to MHC I may occur when dendritic cells take up apoptotic vesicles from infected macrophages [93]–[95].

What is the significance of these results? If B-cells have the ability to induce cytokine production by $CD8^+$ T-cells, then it may induce the production of cytokines and other cytotoxic factors by these cells as well. This would serve as a means to target infected cells to rid the host of the invading pathogens [96]. Vaccines are commonly targeted in a manner which enhances T-cell responses to *M.tb* infection [88], [97]. However, vaccines that target $CD8^+$ T-cell responses are now being considered as well [96]. Thus, if B-cells are able to induce cytokine production by $CD8^+$ T-cells, or $CD4^+$ T-cells, then vaccines can be designed to target B-cells so as to indirectly enhance cytokine production by both $CD4^+$ and $CD8^+$ T-cells. Similarly, once the role of Bregs have been elucidated, including FasL-expressing B-cells, perhaps these cells could also serve as a target for vaccines in order to enhance T-cell responses.

Collectively, these results show that B-cells play a role in modulating T-cell behaviour, whether it be the T-cell phenotypes or effector cytokine production. These results are only preliminary and have a few limitations, including the small sample size and the absence of an active TB disease group but represent our initial attempt to further elucidate how B-cells modulate T-cell behaviour during disease.

Chapter 5. Discussion

TB disease represents a spectrum which include a healthy infected state without the presentation of symptoms (i.e. latent TB infection), and TB disease with symptoms [98]. Much research has gone into understanding how the host immune system responds to invasion by *M.tb*, and how the immune system fights the invasion. However, one has to take into account the different phases of disease in order to understand why some people's immune system can contain the infection and others cannot. These phases include the innate immune phase, during which macrophages are infected with *M.tb,* followed by the adaptive immune phase, during which cell-mediated immunity is initiated through presentation of antigen to T-cells. At this stage, the immune system can either contain the infection, which is termed the quiescent phase or latent infection, or the infection can develop into an active replicating phase which is TB disease [99]. In order to fully understand the multitude of ongoing processes that lead to control of infection or the establishment of immunopathology, or at least attempt to, the focus should be on every aspect of the immune system such as the different immune cells and how they differ in phenotype and function during the different disease states and how it compares to that of uninfected (and exposed) healthy individuals. For the purpose of the study, B- and T-cells were the focus of this study from cohorts which included TB cases followed up throughout treatment, healthy uninfected and healthy infected individuals (latent infection), as well as other lung disease controls. The aims of the study was to a) evaluate how B-cells regulate their genes/receptor repertoire during *M.tb* infection/exposure and how this is affected by anti-TB treatment and b) to investigate how B-cells modulate T-cell behaviour in latently infected individuals compared to healthy uninfected individuals. T-cells, according to research, are at the forefront of the defence against infection with *M.tb*. Some say that multifunctional $CD4^+$ T-cell responses correlate with protection against infection [35], [100], whereas others disagree [101]. On the contrary, B-cells have not been of any

significance in the defence against *M.tb*. Research of B-cells during TB show that humoral responses may enhance protection by improving killing by phagocytes, but is not essential for protection [102]–[104]. However, as the understanding of B-cell development has progressed, it is apparent that B-cell function extends beyond antibody production and antigen presentation. Additional functions include cytokine production and various regulatory phenotypes [20], [21], [83]. Therefore, we proposed that B-cells have the potential to influence T-cell behaviour.

In order to address the first aim, transcriptional and phenotypic analysis was utilised. Transcriptional analysis is useful for several reasons including biological discovery, as well as identifying biomarkers [48], [49], [51]. Evaluating the levels of specific genes expressed by immune cells gives insight into particular functions of the cells [48], [49]. Furthermore, one can utilise phenotypic analysis for biological discovery at the protein level, as a means to confirm results from transcriptional analysis (gene level). Cohorts used for this part of the study included healthy controls with a QFN positive status which is indicative of latent TB infection (LTBI), as well as TB cases who were followed up at various time points throughout anti-TB treatment. In addition, a group of participants with other lung diseases were included as controls. These participants were recruited from areas to which TB is highly endemic, and where more than the chances of individuals having been exposed to *M.tb* is high. Prevalence rates in these areas have been reported as high as 1000/100 000 [105].

The present study had some shortcomings, including small cohort sizes and cohorts recruited mainly from one area (even though high burdened) which should be addressed in future studies; drawing conclusions from these results could lead to over interpretation of significance. Furthermore, the co-culture experiments were only carried out using QFN positive and negative individuals. Although this pilot study represents the first attempt at understanding the complex interactions between B-cells and T-cells in a non-diseased state it would be important to include

newly diagnosed, untreated TB cases and follow them up from diagnosis to successful treatment, as well as other lung disease controls in order to understand the dynamics of the interaction during active disease states.

Firstly, the results from the transcriptional analysis of B-cells from healthy controls and TB cases showed that B-cell genes which is indicative of activation, such as *CD19* and *APRIL* was increased by the end of treatment. These results fit in with a previous report from our group that indicated circulating marginal zone and memory phenotypes of B-cells differ between pre-TB treatment and at the end of TB treatment. That study also found that a sub-population of activated memory B-cells ($CD19^{+}IgM^{+}CD23^{+}CD27^{+}$) are present at the end of successful TB treatment [106]. This result was confirmed by phenotypic analysis of B-cells from healthy controls and TB cases followed up during treatment in the current study. The frequency of B-cells expressing CD40 receptor increased during treatment, which is indicative of B-cell activation. B-cells require two signals for activation, one of which is B-cell receptor ligation and the second signal being ligation of CD40 by CD40 ligand (CD40L) [16]. Therefore, when CD40 ligates, the cell upregulates the expression of CD40 for maximum ligation/activation. These results are in line with a study by Cliff and colleagues (2013) which indicated that there were increased expression of IgM, IgD as well as CD19, later during treatment as shown by transcriptional analysis [48]. This suggests that B-cells are either depleted in the periphery, or that gene expression is altered during TB disease. Thus, our results indicate that anti-TB treatment is inducing B-cell activation as a means to restore the immune system. B-cells may be using the production of antibody to opsonize and in turn enhance phagocytosis, activate complement components (C1q and C2) or the B-cells may be producing cytokines that further enhance cell-mediated immune responses against *M.tb* [107], [108]. Generally, it is understood that antibodies mostly protect against extracellular pathogens,

but evidence exists which suggests that antibodies have the potential to protect against intracellular pathogens such as *Leishmania* species and *Plasmodium berghei* [109], [110].

As previously mentioned, B-cells possess non-humoral functions as well, which includes the production of cytokines. Additionally, there are small subsets of B-cells that are being discovered including regulatory B-cells (Bregs) which produce cytokine such as IL10 and express death-inducing markers such as FasL [64]. These Bregs are mostly researched during autoimmune disease, but evidence lead us to believe that these Bregs may be active during TB as well.

The results from transcriptional and phenotypic analyses showed that B-cell regulatory functions are enhanced during anti-TB treatment. This is indicated by the increase in the expression of *FASL* and *IL5RA*, which are genes associated with a small subset of FasL-expressing regulatory B-cells [64]. This finding was confirmed by phenotypic analysis of B-cells from TB cases at diagnosis and throughout treatment, where the results indicate that there is an increase in the frequency of B-cells expressing FASL and IL5RA by the end of treatment. To further confirm this increase in expression of FasL, BAL fluid from TB cases during treatment and controls was analysed by means of Luminex technology. This serves as a proxy for the site of infection. The results showed that there was an increase of sFasL in the lung of TB cases at the end of treatment. Although one cannot directly conclude that the B-cells in the lung are producing the sFasL, literature does suggest that there is an infiltration of B-cells into the lung during inflammation [69]–[72]. Thus, B-cells are potentially contributing to the sFasL levels in the lung. Collectively, these result indicate that anti-TB treatment boosts B-cell activation, as well as Breg activity. More specifically, there is an increase in the activity of killer B-cells. These $CD19^{+}IgM^{+}$ FasL-expressing B-cells have been studied during autoimmune disease and parasite infections, and the results showed that these cells are induced by stimulation with CD40L and IL5, and that it induces FasL-mediated apoptosis of $CD4^{+}$ T-cells [64]. These cells may have a protective or detrimental

role depending on the disease condition. For instance, during autoimmune disease, apoptosis of $CD4^+$ T-cells reduces auto-inflammation. In contrast, apoptosis of Th1 cells may cause a skewed balance between Th1 and Th2 cells, leading to airway inflammation and asthma due to cytokines produced by Th2 cells. In the current study, however, the increased expression of FasL during successful anti-TB treatment implies that these FasL-expressing B-cells may have a protective role. One hypothesis is that these cells may be inducing apoptosis of infected T-cells as a means to clear bacterial infection, or they may be inducing activation of T-cells which subsequently lead to apoptosis (Figure 5.1). The latter hypothesis has been termed activation-induced cell death [77], [111]. Further studies are required to elucidate this phenomenon.

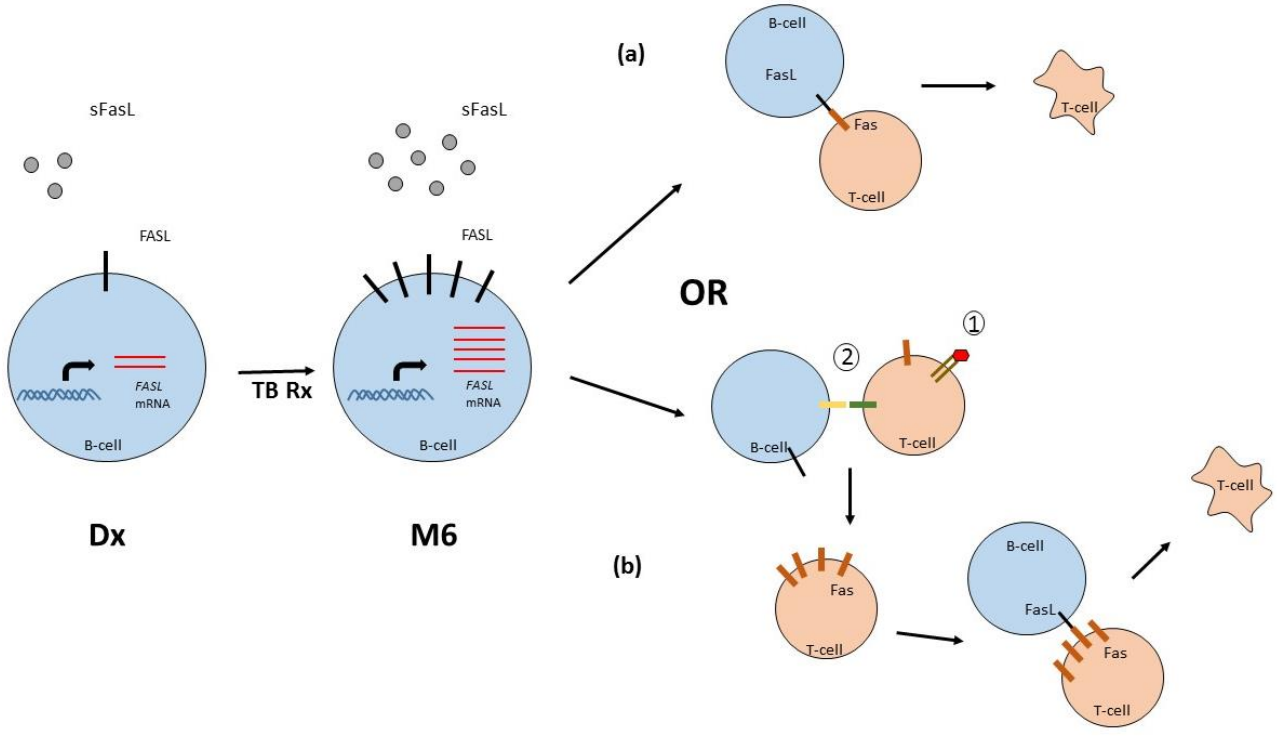


Figure 5.1. Proposed effect of FasL-expressing B-cells during active TB disease
The diagram represents possible interaction scenarios between FasL-expressing B-cells and T-cells. There is an increase in the expression of FasL mRNA and surface-expressed FasL by the end of anti-TB treatment, where Dx is at diagnosis and M6 is end of treatment. A proposed hypothesis for the effects of these B-cells is: **(a)** B-cells directly induce FasL-mediated apoptosis of T-cells or **(b)** T-cells may undergo activation, where 1 represents TCR ligation and 2 is co-stimulation, which leads to an increase in the expression of Fas by T-cells. Thus, making them susceptible to FasL-mediated apoptosis.

Seeing that B-cells have these additional functions, we hypothesised that the extent of the relationship between B- and T-cells spreads beyond providing co-stimulatory signals to one another via co-stimulatory molecules that are expressed by the respective cells. In order to investigate this extended relationship, autologous B- and T-cells from *M.tb* exposed and unexposed (as defined by QFN status) individuals were co-cultured under different antigenic conditions. Subsequently, B-cell phenotypes were assessed, as well as T-cell phenotypes and cytokine production by means of flow cytometry.

Breg frequencies were higher in QFN negative individuals compared to QFN positive individuals, but not significantly higher. As previously mentioned, another Breg subset exists that expresses FasL and induces apoptosis of $CD4^+$ T-cells. Here, we found that BCG in combination with CD40L and IL5 induces an increase in the frequency of FasL-expressing B-cells.

Assessing Treg frequencies showed that B-cells that were stimulated with BCG, and CD40L and IL5, induced a decrease in the frequency of Tregs in both *M.tb* exposed and unexposed. Furthermore, unstimulated B-cells induced an increase in the frequency of TEs in *M.tb* exposed individuals. On the contrary, B-cells stimulated with BCG, and CD40L and IL5, induced a decrease in the frequency of TEs from *M.tb* exposed individuals. The decline in TEs may be due to the increase in FasL-expressing cells, as Klinker and colleagues also showed that these FasL-expressing B-cells induce apoptosis of $CD4^+$ T-cells [64].

Research shows that Bregs induce Tregs and suppress TEs during autoimmune disease [83]. Thus, this relationship between Bregs, Tregs and TEs was assessed in the current study. There was a significant positive correlation between Bregs and Tregs from *M.tb* exposed individuals that have been stimulated with CD40L and IL5. Similarly, there was a significant positive correlation between Bregs and TEs from *M.tb* exposed individuals that have been stimulated with CD40L and IL5 (Figure 5.2). However, this differs from the proposed negative relationship between Bregs

and TEs. This may be because of the different conditions (i.e. *M.tb* exposure vs autoimmune disease), or it may be due to the small number of participants analysed as part of the current study. Furthermore, the significant correlations in only *M.tb* exposed individuals implies that prior or ongoing exposure to *M.tb* may have altered the activation status or recognition by the immune system.

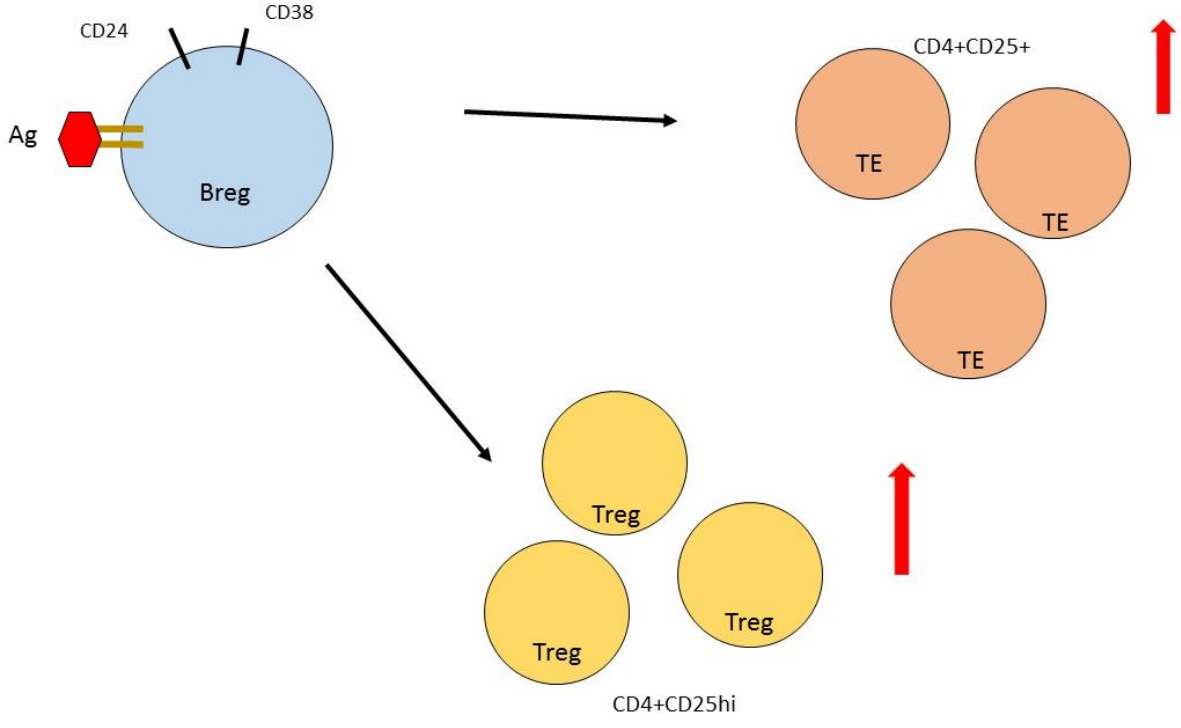


Figure 5.2. Proposed relationship between Bregs, Tregs and TEs during LTBI
The diagram represents a proposed relationship based on the results from the current study. The correlation results imply that antigen activated $CD19^{+}CD24^{hi}CD38^{hi}$ Bregs induce $CD4^{+}CD25^{hi}$ Tregs as well as $CD4^{+}CD25^{+}$ TEs. Where Ag (red) is antigen, Bregs (blue) is regulatory B-cells, Tregs (yellow) is regulatory T-cells and TEs (brown) is effector T-cells.

The enhancement of Tregs have different effects, depending on the condition. For instance, if Tregs are enhanced during autoimmune diseases, it suppresses or dampens the inflammatory responses by effector T-cells [83]. However, during TB disease, enhanced Treg frequencies may have a detrimental effect. A murine study has shown that depletion of Tregs in mice which have been infected with *M.tb* displayed better clearance of bacteria [86]. Further studies indicated that Tregs from individuals with active TB disease dampen T-cell responses to *M.tb* antigen [26], [27]. A study by Shafiani and colleagues (2010) further confirmed that there is an increased bacterial load in mice when there is enhanced Treg frequencies, and attributed this to Tregs' ability to delay effector T-cells to migrate to the lung [87]. The latter effects are mostly attributed to $CD4^{+}CD25^{hi}FoxP3^{+}$ Tregs, and the current study focussed on $CD4^{+}CD25^{hi}$ Tregs. Further studies are required to confirm whether these cells are the same.

Assessing cytokine production by $CD4^{+}$ and $CD8^{+}$ T-cells indicated that B-cells on its own, or following stimulation with BCG, and CD40L and IL5, are able to induce cytokine production by both T-cell phenotypes. These results are from healthy individuals who have either been exposed to *M.tb* or not. It is important to know what occurs in a "healthy" state, so that one can understand how the immune system resists or contains infection by invading pathogens. Here, studying the immune response of latently infected/exposed individuals is essential, as most of the population are latently infected. Also, prior work from our group on individuals from the same high TB burden area showed that B-cells are able to produce cytokine in response to BCG stimulation or TLR9 activation in latently infected/exposed individuals [106]. The cytokine production was mostly attributed to B-cells with a plasmablast ($CD138^{+}$) phenotype [112]. Similarly, Bermejo and colleagues identified B cells with a plasmablast phenotype as a major source IL17 production in response to *Trypanosoma cruzi* infection [29].

Collectively, the results imply that B-cells affect T-cell phenotype and function. However, the questions remain: why are these results significant or what impact will the knowledge of B-cell function have on TB research? This is important, because most vaccines are aimed at enhancing T-cell responses in order to improve protection against *M.tb* infection [97], [113]. If TB vaccines are aimed at enhancing T-cell responses, then these results offer a potential alternative to inducing or enhancing T-cell responses. Here, the results indicate that B-cells also induce cytokine production by $CD4^+$ T-cell as well as $CD8^+$ T-cells. This is interesting as Pinxteren and colleagues (2000) found that $CD8^+$ T-cells are required for the control of *M.tb* infection in latently infected mice [91]. There is also evidence that $CD8^+$ T-cells play a role during active TB [90], but further studies are required to confirm this in humans. Thus, if B-cells are able to induce cytokine production by $CD8^+$ T-cells, then perhaps it may induce the production of cytokines and other cytotoxic factors by these cells as well. This would serve as a means to target infected cells to rid the host of the invading pathogens [96]. Similarly, once we have further elucidated the role of the FasL-expressing B-cells, and why exactly they are being induced during treatment, one could aim to improve current treatment regimens to further enhance the frequency of these B-cells, or design a vaccine that targets FasL-expressing B-cells in order to protect against *M.tb* infection.

Additional studies are clearly required to further elucidate the relationship between B- and T-cells, and how exactly B-cells are inducing T-cell responses, and to what extent the responses would be beneficial. For instance, are B-cells using cytokines (e.g. IFNγ or IL10), surface molecules (e.g. FasL) or a combination of both to modulate T-cells responses? Also, more understanding of signalling pathways leading to B-cell activity are required. For instance, transcriptional analysis from the current study indicated an increase in the expression of APRIL. Is this a potential target for a vaccine or treatment? Enhancing the expression of APRIL may enhance B-cell activation and subsequent effector functions. Once we have a better understanding of this, vaccines may be

designed to target B-cells in a manner so as to indirectly modulate $CD4^+$ and $CD8^+$ T-cell responses in order to protect against *M.tb* infection and disease.

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Addenda

Addendum A

Table A1. Demographics of Cohort used for B-cell phenotypic analysis

	TB	CTRL
No. of Females	2	10
No. of Males	11	5
Median Age (years old)	50	38
Gene Xpert-MTB/RIF Assay	POSITIVE	NEGATIVE
QuantiFERON status	NA	POSITIVE
Sputum-culture status (Dx)	3 NEGATIVE 10 POSITIVE	NA

Table A2. Demographics of Cohort used for Gene expression analysis

	TB	CTRL	OLD
No. of Females	8	10	5
No. of Males	12	0	5
Median Age (years old)	28	24	37
QuantiFERON status (Dx)	NA	POSITIVE	5 POSITIVE 5 NEGATIVE
Sputum-culture status (Dx)	POSITIVE	NEGATIVE	NEGATIVE

Table A3. Demographics of Cohort used Luminex analysis

	TB (Dx)	CA	TB (M6)
No. of Females	4	7	9
No. of Males	4	3	11
Median Age	41	60	31
Gene Xpert/Sputum-culture	POSITIVE	NEGATIVE	NEGATIVE

Addendum B

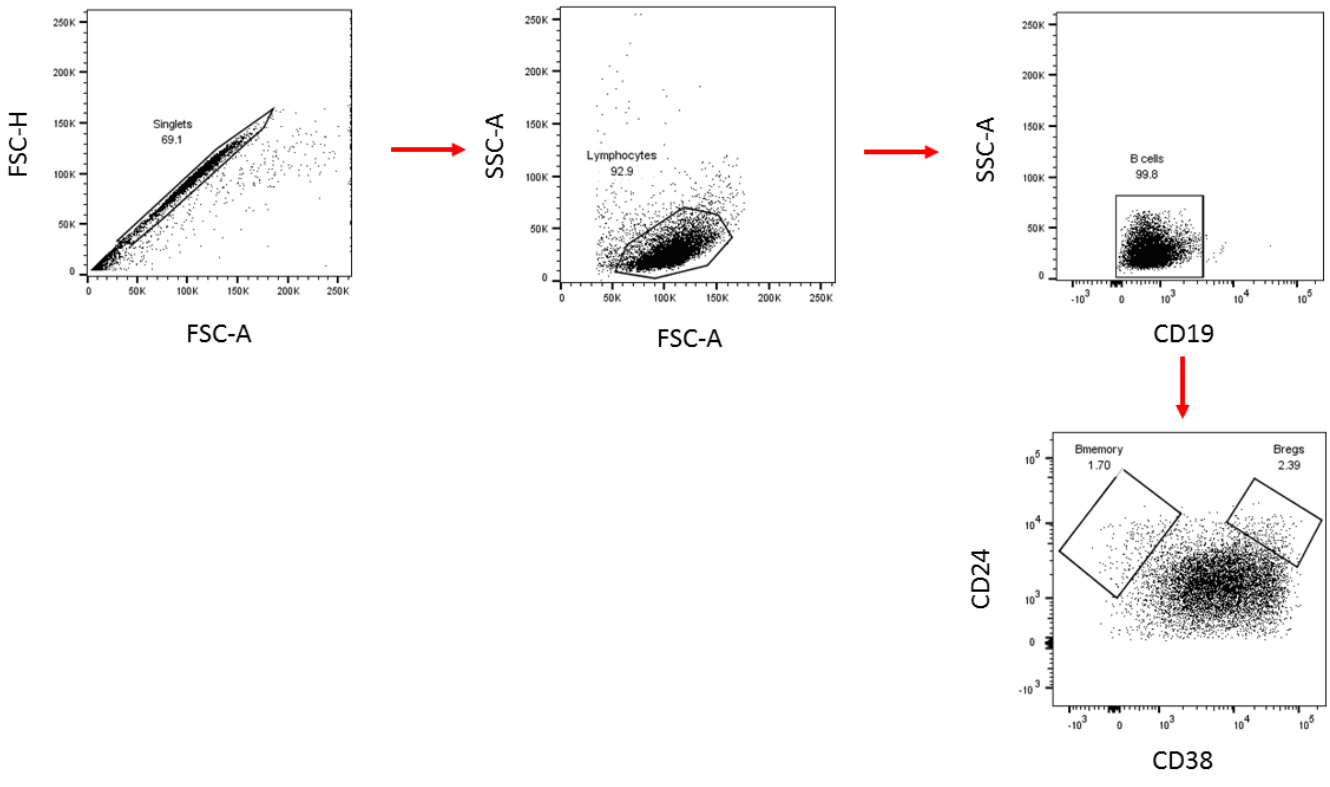

Figure B1. Gating strategy for identifying Breg and Bmemory cells.
Single cells were gated to exclude doublets, and subsequently lymphocytes were gated on SSC and FSC. $CD19^{+}$ B-cells were included in order to identify $CD24^{hi}CD38^{hi}$ Bregs and $CD24^{hi}CD38^{-}$ Bmemory cells. Cut-off values were obtained using stimulated T-cells from PBMCs.

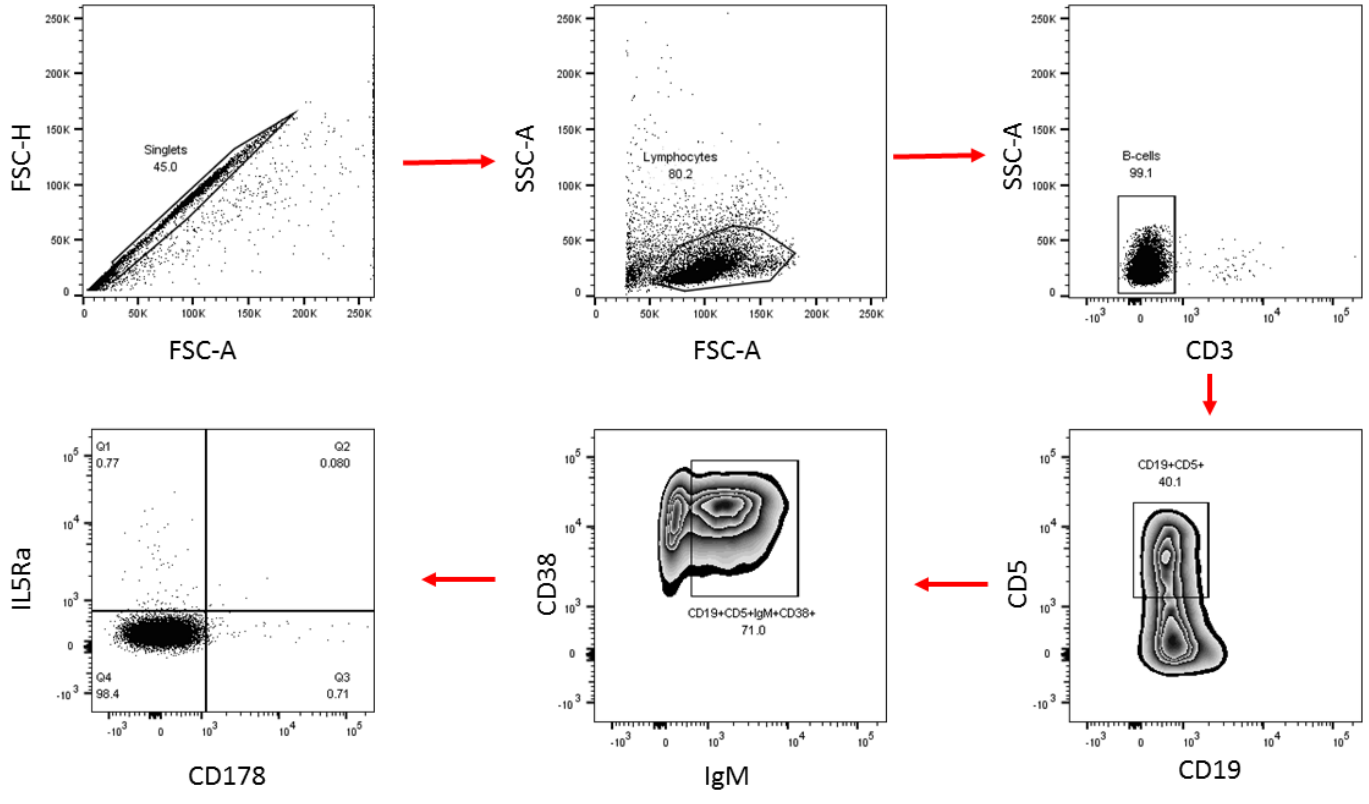

Figure B2. Gating strategy used to identify FasL-expressing B-cells
To identify FasL-expressing B-cells, lymphocytes were gated on SSC and FSC. $CD3^-$ lymphocytes were included for further analysis. Subsequently $CD19^+CD5^+$ B-cells and IgM^+CD38^+ cells were gated in order to assess the expression of CD178 and IL5Ra. The cut-off values were obtained using stimulated T-cells from PBMCs.

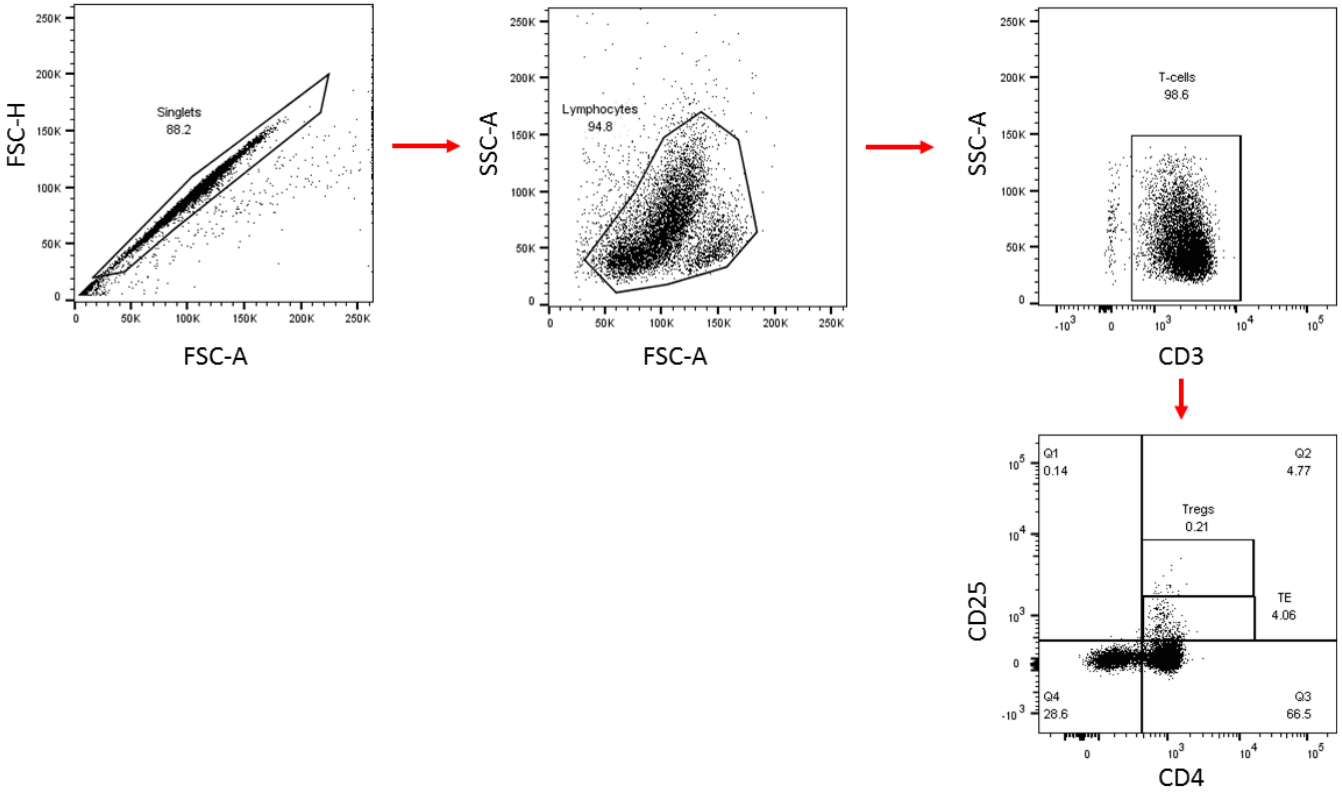

Figure B3. Gating strategy used to identify Treg and TE cells
To identify Tregs and TEs, lymphocytes were gated on SSC and FSC and $CD3^{+}$ lymphocytes were included for further analysis. Subsequently $CD4^{+}CD25^{+}$ TEs and $CD4^{+}CD25^{hi}$ Tregs were gated. The cut-off values were obtained using stimulated T-cells from PBMCs.

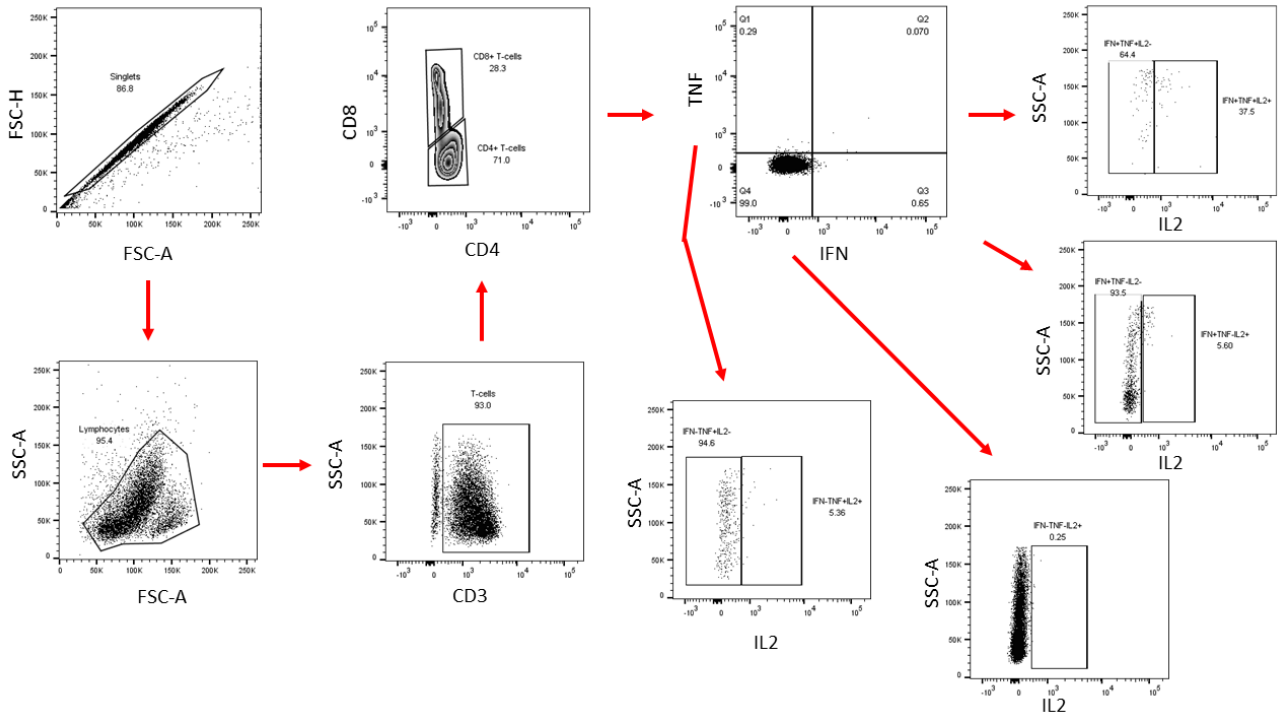

Figure B4. Gating strategy used for $CD4^+$ and $CD8^+$ T-cell cytokines
To identify cytokine-producing T-cells, lymphocytes were gated on SSC and FSC. $CD3^+$ lymphocytes were included for further analysis. Subsequently, $CD4^+$ and $CD8^+$ T-cells producing different combinations of IFNγ, TNFα and IL2 were gated. The cut-off values were obtained using stimulated T-cells from PBMCs.